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NATIONAL INSTITUTE OF ENVIRONMENTAL HEALTH SCIENCES
Fiscal Year 1975

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OFFICE OF THE DIRECTOR

OFFICE OF THE DIRECTOR
Summary Statement

The heart of the annual report of an NIH Institute is the collection of project reports developed by each intramural scientist. These reports represent each scientist's scientific accomplishments and aspirations. NIEHS' excellent reports have been summarized in reports by each Branch Chief. Additionally, each Associate Director has described those program activities beyond the intramural laboratories. Therefore, I shall not comment on them here. Instead, I want to take this opportunity to comment upon the state of our environmental health and where NIEHS fits into the National effort to cope with the resulting problems.

New forms of energy production, expanded uses of known energy sources, greater development of the chemical process industry and, particularly, the petrochemical industry (the United States now reproduces the body weight of each American citizen in plastics each year), all pose the real threat of releasing toxic chemicals into the environment.

Recent experiences with vinyl chloride, bischloromethyl ether, methyl butyl ketone, and sulfuric acid mist from exhausts of 1975 automobiles indicate that these compounds are not theoretical threats but known causes of mortality and morbidity.

The current breadth of NIEHS program activities is illustrated in the subjects of the project reports. These range from the respiratory toxicity of antiperspirants and red tide, through the mechanism and comparative pharmacology of epoxide formation of environmental chemicals, to the effects of prenatal exposure to diethylstilbestrol (DES) on the reproductive development of mice, the interaction of acetylaminofluorine (AAF) with DNA repair synthesis, the development of spot tests for mutations in *Neurospora*, the effects of non-ionizing radiation on quail egg development, and the construction of models to permit extrapolation from the results of exposure of small populations of animals to high doses of chemicals to the expected results of exposure of large populations to small doses of these chemicals.

As we approach the end of the first decade of the Institute's existence, it is clear that the Nation's problems in environmental health have grown in number, intensity, and variety. Additionally, it is clear that our view of our mission must grow if we are to continue to be responsive to the expanding National need in this area.

NIEHS must perform the biomedical research necessary to understand mechanisms of toxicity; to identify susceptible and resistant subsets of the population; to develop, validate, and interpret new methods to detect toxicity; and, in general, to provide the bridge between basic biomedical research characteristic of the typical NIH laboratory and applied-test-oriented research carried out under sponsorship of regulatory agencies. NIEHS, with its limited rented laboratory space and its limited personnel, is not in a position to perform this vital function in a quantitatively satisfactory manner today. The proliferation of new chemical compounds makes it mandatory that NIEHS be properly supported so that it can keep pace with the continuing industrial assault on public health.

OFFICE OF THE ASSOCIATE DIRECTOR
FOR INTERAGENCY PROGRAMS

OFFICE OF THE ASSOCIATE DIRECTOR FOR INTERAGENCY PROGRAMS
Summary Statement

During the past year, this Office has played the lead role on behalf of the Office of the Director, NIEHS, in two areas: US-USSR Cooperation and Energy-Related Research.

US-USSR Cooperation

Collaboration between Soviet and American environmental health scientists is being carried out under the aegis of two cooperative agreements between the United States and Soviet Union. Under the terms of the *Medical Science and Public Health Cooperative Agreement*, scientists from both countries are committed to regular, long-term cooperative efforts in the areas of heart disease, cancer, arthritis, influenza, and acute respiratory diseases, and health problems associated with the environment. The Director, NIEHS, is US Coordinator for the environmental part of the Health Agreement.

1975 was the third year of formal cooperative efforts in environmental health between the US and USSR. The first year was concerned largely with establishing working relationships and agreeing on areas for joint study. Initiation of joint research efforts began in the second year of the Agreement and involved exchange visits between scientists of both sides. In December 1974, American and Soviet researchers participating in the Agreement met at the invitation of the Soviet government in Riga, Latvia, to present the results of their cooperative effort to date. The results of this symposium will be published in 1975 in both the United States and the Soviet Union.

A cooperative research program for 1975-1976 was agreed upon during the Riga symposium. Research efforts are divided into three problem areas which seek to explore the methodological basis for assessing the biological effect of chemicals which might be inhaled, or introduced orally, or both simultaneously.

By the end of 1975, over twenty scientific papers will have been published by American and Soviet scientists on environmental health research conducted under the auspices of this Agreement.

An initial draft of a Russian-English glossary of environmental health terms was prepared during 1974 and is currently under review by both sides. A first edition of this glossary will be published in 1975 in both countries.

The *Agreement on Cooperation in the Field of Environmental Protection* between the US and USSR is aimed at addressing the most important aspects of problems of the environment, including the study of pollution and its effects on the environment. A number of agencies participate in this Agreement which is

under the general direction of the Administrator, EPA. The Director, NIEHS, serves as HEW representative to the Environmental Protection Agreement and as Co-Chairman of the working group for one of the areas of the Agreement, the Biological and Genetic Effects of Pollutants. Effort in this area has been focused on the mutagenicity of environmental contaminants, the toxic effect of heavy metals in the environment and epidemiological studies. Program activity in 1975 consisted largely of information exchange and a joint workshop in New York City in April on "Basic and Practical Approaches to Environmental Mutagenesis and Carcinogenesis".

Energy-Related Research

As part of the high priority accorded by the Federal Government to solving the energy problems facing the nation, the Director and staff of NIEHS have participated in a variety of activities focused on elucidating the potential adverse health impacts of various energy technologies. During the past year the Director, NIEHS, Co-Chaired a CEQ/OMB task force to advise the Executive Office on the allocation of funds (which were included in the FY 1975 budget) for research on the potential adverse health effects of energy extraction, processing, utilization, and conservation. As a result of the work of this task force, NIEHS was assigned the responsibility, and given additional funds, to develop research programs to address a number of important energy-related questions, including the development of physiological indicators to estimate damage to man; the determination of mechanisms of incorporation, metabolism, deposition and turnover of hazardous agents; the determination of the relationship of metabolism and fate and the toxicology of particulates and organic compounds; the determination of the dose-effect relationship for physiological effects; the determination of the dose-effect relationship for mutagenic agents; the development and testing of models for extrapolation of cellular and animal data to man; the identification of damage to cells and cell components; and the determination of immediate and long-term effects of critical pollutants on selected ecosystems and organisms in the marine environment.

This Office is responsible for coordinating the Institute's role and relationships with other agencies involved in energy-related research.

OFFICE OF THE ASSOCIATE DIRECTOR FOR PROGRAM

OFFICE OF ASSOCIATE DIRECTOR FOR PROGRAM
Summary Statement

During Fiscal Year 1974 the Office of the Associate Director for Program continued its activity in surveillance of potential health hazards of technological developments with the help of a Chemical Engineer, a Pharmacologist, and recently a Biochemist.

Several studies were carried out in depth to focus on the potential health hazard from augmentation and changes in energy production, either real or projected, involving various fuels. A shift to methanol as a useful and easily obtainable fuel for automobiles was given some emphasis and the toxicity of methanol was stressed and documented.

Following the observations that vinyl chloride was a potent carcinogen, a number of chemicals were taken under scrutiny because of their close chemical relationship to vinyl chloride and potential or already established carcinogenic potency. These compounds are still in use and this may represent a hazard. Amongst them, vinyl bromide deserves instant attention because it is likely to behave in a similar manner to vinyl chloride as a carcinogen, and its use has been encouraged as a fire retardant. Several laboratories have been interested by this office in testing the compound for carcinogenicity.

Interest has also been maintained in the assessment of other fire retardants such as mirex because of the expressed priority to protect the population from burns by flammable garments and furnishings. However, the toxicity of the fire retardants and their pyrolysis products need careful assessment.

The importance of chlorine as a free radical reducing the ozone layer in the stratosphere and thus allowing increased amounts of ultraviolet and infrared radiation reaching the earth has been given a great deal of public attention, and this office has represented HEW in an interdepartmental effort to determine the hazard of the release of freons and other chlorine containing compounds that might contribute to this destruction of the ozone layer and the potential cancer hazard to the skin of man as well as other environmental adverse effects.

During this year a member of this office has participated in a program ranking industrial chemicals with regard to their potential health impact based on the probability of reaching the environment in quantity and possessing toxic effects. This program is designed to facilitate decision making regarding potential harm to man now or in the future.

Recently the timely addition to the staff of this office enabled us to expand the surveillance activity to initiate reviews of cataractogenic chemicals in the environment and to coordinate with other scientists inside and outside of NIEHS an assessment of the state-of-the-art of detecting developmental behavioral toxicity due to environmental influences.

Last fiscal year the Associate Director for Program was detailed to WHO to help establish there a program of international cooperation in preparing health criteria documents on environmental pollutants. He also initiated a program to assess potential health hazards from new technological developments. Both these programs are now ongoing. The former produces documents assessing health hazards of important toxic elements and organic chemicals. This activity will ultimately produce health criteria documents for all important toxic agents, chemical or physical, that man may be exposed to. In the latter program, assessing the health hazards of new technological developments, cooperation of NIEHS with WHO will produce a second meeting at this Institute early next fiscal year on health hazards due to chemicals used in the rubber and plastics industries which is now in preparation.

Other cooperation activities of this office with international agencies consisted of participation in a meeting in London of the joint group of experts on the scientific aspects of marine pollution (GESAMP) where an assessment of the hazard to man and the environment of pollution of the ocean by metals and organic chemicals was carried out. This international activity hopes to cover in the future all chemicals that may present a hazard to all life in the ocean and to man through the food chain or in his use of the ocean.

The Associate Director also represented WHO at the meeting on impact of production and use of energy on the environment sponsored by the United Nations Environmental Program (UNEP). He is also preparing for WHO the chapter on "Modulating factors and isolated systems" in a Manual on the evaluation of the toxicity of chemicals.

Another cooperative enterprise is being carried out with the International Agency for Research on Cancer (IARC) on the carcinogenicity of natural products which is being documented at this time for presentation to an expert committee to be convened in Lyon, France, early next fiscal year.

The Associate Director cooperated with the HEW Toxicology Subcommittee for carcinogen standards advising on problems regarding the protection of laboratory workers from contact with carcinogenic agents.

For the better evaluation of potential health hazards this office has helped HEW in the assessment of drafts on Effluent Limitations Guidelines and Environmental Impact Statements prepared by a number of agencies. This activity turned out to be exceedingly time consuming and unfortunately there was usually no evidence that these comments which attempted to be of help to the agencies were acknowledged or heeded. Hopefully even better understanding can be reached in the future between HEW and the other agencies in this respect.

Representing HEW the Associate Director was asked to participate in contract assessments for the U.S. Army Medical Research and Development Command to evaluate potential contractors as well as the protocol and research aims to be activated.

Cooperation with the National Cancer Institute has always been a function of this office and the Associate Director participated as a panel member in the evaluation of the carcinogenicity of the detergent builder nitrilotriacetate, took part in the NCI-chaired meeting of the committee on environmental hazards (American Academy of Pediatrics) and serves as a consultant to the National Cancer Advisory Subcommittee on environmental carcinogenesis.

On invitation by the American Physiological Society, the Associate Director prepared a chapter for the Handbook of Physiology on "Nature, Origin and Distribution of Chemical Agents -- Smoking" and recently took on the responsibility to help as associate editor in facilitating the publication of this volume.

He also assisted in the review of the draft of the 1975 report to Congress on the Health Consequences of Smoking for the Center for Disease Control.

In order to establish good liaison and collaboration with the National Center for Toxicologic Research (NCTR), the Associate Director visited the Center on invitation of its director to familiarize himself with their program.

On invitation by the Environmental Protection Agency (EPA), the Associate Director participated in a Conference on Water reuse, chairing the Panel on Organic Pollutants. In this problem area of how to assess the hazard of organic pollutants in drinking water and how to test for toxicity, the Associate Director is preparing a presentation to be given in Aspen, Colorado, later in the year.

The Associate director participated as a member of several committees of the National Academy of Sciences (NAS), Committee on Biological Effects of Ionizing Radiation (BEIR)-Subcommittee on Energy, the Committee on Medical and Biologic Effects of Environmental Pollutants (MBEEP)-Vapor Phase Organic Pollutants Panel, and as representative of NIEHS to the Medical and Biologic Effects of Environmental Pollutants Committee (MBEEP) to keep abreast of their activities and advise on areas of concern.

The Associate Director remained active on the editorial board of several journals, including the Environmental Health Perspectives (EHP), the Journal of the National Cancer Institute, the Chemico-biological Interactions, and others as requested.

1. Associate Director for Program
- 2.
3. Research Triangle Park, N.C.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

PROJECT TITLE: "An Investigation of Environmental Pollutants Causing Porphyria"

PREVIOUS SERIAL NUMBER: NIEHS-ADP-001

PRINCIPAL INVESTIGATOR: Hans L. Falk, Ph.D.

OTHER INVESTIGATORS: Sandra Jordan

COOPERATING UNITS: None

MAN YEARS

Total:	1.1
Professional:	0.1
Other:	1.0

PROJECT DESCRIPTION

OBJECTIVES: To determine the porphyrogenic properties of certain environmental chemicals in rats and to assess the degree of porphyria produced in individuals, the duration of the effect and the recovery following cessation of treatment.

METHODS EMPLOYED: Oral intubation of the chemical or feeding to young or mature rats. Progress of the metabolic disorder is studied by fluorescence and quantitated by UV absorption spectroscopy of urine samples.

MAJOR FINDINGS: Hexachlorobenzene, a rather common environmental pollutant, causes long lasting porphyria in rats when administered in the diet for a few days. Animals given the chemical will respond to similar degree in showing porphyria, but their recovery may be quite different for similar individuals. In certain individuals it may disappear at a steady rate over several months, while in others recovery will be very slow and incomplete. Subsequent exposure to the porphyrogen will produce an increase in porphyria in all animals, but the recovery will again be of the same type as on previous exposures, typical for the individual. The fluorescent compounds detected in the urine, copro- and uroporphyrin, are not produced in the same proportion with repeated hexachlorobenzene feeding.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: In toxicological research the expectation is expressed that similar treatment of

animals specified with regard to strain, sex, nutrition, and other important parameters will produce similar effects. In all biological studies a certain degree of variation of response is of course expected and can be taken into account. However, when part of the response -- in this case the recovery -- seems to fall into two separate classes, it becomes necessary to follow up these observations to determine those factors responsible for the difference in response.

PROPOSED COURSE: Rats of the same strain are to be given hexachlorobenzene or specific hexachlorobiphenyls which are porphyrogenic to induce porphyria at certain intervals and to study the degree of toxicity as well as the rate of recovery. Factors that may influence the rate of recovery will be investigated.

KEYWORD DESCRIPTORS: fungicide, porphyrogen, porphyria.

1. Associate Director for Program
- 2.
3. Research Triangle Park, N.C.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

PROJECT TITLE: "Technology Surveillance"

PREVIOUS SERIAL NUMBER: NIEHS-ADP-002

PRINCIPAL INVESTIGATOR: Warren T. Piver, Ph.D.

OTHER INVESTIGATORS: Hans L. Falk, Ph.D. and Herbert S. Posner, Ph.D.

COOPERATING UNITS: Institute-wide

MAN YEARS

Total:	1.0
Professional:	1.0
Other:	0.0

PROJECT DESCRIPTION

OBJECTIVES: The objectives of this program are: 1) to stay abreast of present and developing technologies related to the production and use of chemicals; 2) to examine the sources, rates of release, methods of transport, and potential levels of exposure to hazardous chemicals; 3) as a result of objectives 1 and 2, to suggest research needed to provide a firmer scientific basis for regulation of hazardous materials; and, 4) to suggest modifications in chemical plant design, product design, use pattern, or disposal practice to minimize adverse environmental effects.

METHODS EMPLOYED: The methods used in this program are: 1) literature review aimed at synthesis of available information on chemical processes, losses of material during processing, production and disposal of by-products, uses of manufactured chemicals, trends in usage, recognition of structurally similar chemicals, and biological investigations conducted on these chemicals; 2) membership on panels and committees concerned with environmental health aspects of chemicals; and, 3) continual upgrading and refinement of skills in chemical engineering science so that suggested modifications in processes and practices will reflect the latest knowledge in dealing with these problems.

MAJOR FINDINGS: A. The Trace Organic Chemicals in the Environment Panel sponsored by NSF has determined a methodology for ranking industrially important chemicals. This ranking is based on estimated release rate to the

environment, and is a function of production and use volume and that fraction which is lost to the environment in production and use. The list has been narrowed to 85 chemicals. Currently information is being collected on transport, biodegradation, bioaccumulation, autoxidation potential, half-lives in different phases, and mammalian and human toxicity studies. At the present time, it is evident that there is a great paucity of data on many of these subjects. In this final list of 85 chemicals, care was taken to include members of each class or structural group of organic chemicals.

B. Reviews of EPA Effluent Limitation Guidelines and EIS for the Office of the Assistant Secretary for Health, DHEW, have shown that these documents have a consistent lack of understanding of rational pollution control strategy and environmental impact of proposed projects. Consistent suggested changes have been: 1) better characterization of waste streams; 2) segregation of problem wastestreams; 3) more attention to occupational health impact; and 4) more attention to solid waste disposal practices.

C. Vinyl bromide has been suggested for more complete toxicity evaluation. This compound is being produced as a flame retardant monomer and is structurally similar to vinyl chloride. At the present time, very little is known about the mechanism of toxicity of this compound.

D. As a result of Federal legislation, the number of chemicals being used to retard fires is increasing. In many instances, the mechanism of retardation, the nature of the combustion products, and the effects of exposure to these substances is either unknown or poorly understood.

E. Production of energy for the next 25 years will continue to be for the most part, from fossil fuels. Therefore environmental health problems will continue to be from gaseous and particulate air pollutants, solid wastes and heat. All of these substances are by-products from combustion of fossil fuels. Occupational health problems associated with mining of coal will continue. Environmental contamination of oceans and rivers from crude oil and crude oil fractions will continue and may increase as pressure for increased off-shore drilling for oil increases. The rate of release of these substances and the so-called "carrying capacity" of effected ecosystems is poorly understood. Production of a crude oil-like substance from oil shale produces a spent shale with a high content of polynuclear aromatic hydrocarbons. The solid waste practices for this spent shale requires more analysis.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: These surveillance and liaison activities will continue to provide necessary information about products and use patterns of chemicals, important environmental transport mechanisms, and development of methods to predict exposure levels for environmental chemicals. Such information can be used in planning research programs for the Institute.

PROPOSED COURSE: This work will continue and will expand into other areas of interest. This program will attempt to include more and more Institute personnel and representatives from other disciplines and background.

KEYWORD DESCRIPTORS: Technology surveillance; chemical process industries.

PUBLICATIONS

Piver, W.T.: Methods proposed to achieve air quality standards for mobile sources and technology surveillance. Environ. Health Perspec. 10: May 1975.

1. Associate Director for Program
- 2.
3. Research Triangle Park, N.C.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

PROJECT TITLE: "Preventive Surveillance of Environmental Chemicals for Toxic Potential"

PREVIOUS SERIAL NUMBER: NIEHS-ADP-004

PRINCIPAL INVESTIGATOR: Herbert S. Posner, Ph.D.

OTHER INVESTIGATORS: Hans L. Falk, Ph.D., Insu P. Lee, Ph.D., George W. Lucier, Ph.D., Judson W. Spalding, Ph.D. and James P. Woods, Ph.D.

COOPERATING UNITS: Environmental Toxicology Branch, NIEHS; Toxicology Information Research Center, Oak Ridge, Tennessee.

MAN YEARS

Total:	1.5
Professional:	0.3
Other:	0.2

PROJECT DESCRIPTION

OBJECTIVES: The project develops several modes of preventive surveillance and research appropriate to NIEHS. The program assists the chemical process industries and Federal agencies with a regulatory mission in their attempts to minimize toxic events in general or limited populations. The information that is developed relates first to the compounds being considered and then, as greatly as possible, to related chemical or biological situations.

METHODS EMPLOYED: Searching of the literature, consultations with appropriate individuals, consideration of known structure-activity relationships, attendance at meetings, preparation of reports, discussions with those who might assist in further laboratory or theoretical considerations, feedback and dissemination of the appropriate findings and preparation of manuscripts for publication.

MAJOR FINDINGS:

Aminimide compounds: Last year's Annual Report described four reports prepared by this office concerning a large family of compounds with a relatively new functional group, N-N-R; for which little biological information exists.

Limited tests were performed at NIEHS with two highly purified compounds. One caused cell lysis in culture at very low concentrations. Limited or no activity occurred in other tests. Despite continued advertising of the series, in which the statement "significant data is being accumulated regarding solubility, compatibility, stability, toxicity and reactivity" was used, correspondence brought the reply that "the aminimide work is now in a 'holding' position and we really have not developed any significantly new information on the subject since our last correspondence." An abstract appeared recently reporting that some of the compounds are broad-spectrum antibiotics against gram-positive and negative bacteria and against yeasts. Proposals to the company and to NIEHS for a conference have brought negative responses so far.

Compounds with vinyl halide or precursor vinyl halide substructures: The literature was searched for compounds that contain a vinyl halide or potential vinyl halide substructure in the classes of pesticides, drugs, solvents, chemical intermediates, microbial products, food products and chemicals for other uses. The 180 compounds identified were divided into eight groups of varying forms of analogy. Preliminary reports outside NIEHS now suggest that several of the compounds, vinylidene chloride, trichloroethylene and chloroprene, might be showing some evidence of carcinogenic action. A manuscript for publication is being prepared.

Methanol: Methanol is currently being proposed for a large number of uses. The well-known toxicities and hazards, however, are ignored or considerably understated. This condition was brought to the attention of the Director of NIEHS and then the DHEW Toxicology Coordinating Committee. A manuscript has been accepted for publication.

Qualitative and quantitative aspects of structure-activity relationships: The literature on the qualitative and quantitative aspects of structure-activity relationships was reviewed. While much has been accomplished to relate structure and activity quantitatively within particular series of structures, less has been done for smaller series or to relate compounds in different series. Substructural analysis, pattern recognition and side effect sorting are some of the techniques that will be useful in this area.

Outside activities: Meeting attendance: Two of the meetings attended dealt with aspartame, a new sweetening agent, and fiberglass. Reports of these meetings were prepared and circulated to appropriate individuals.

Outside activities: IMOS Task Force: As NIH representative to this Task Force, I have been taking part in considering the response of the Federal Government to the increasing number of reports of chemicals that might be bringing about inadvertent modification of the stratosphere (IMOS). A report is in preparation and is scheduled for release at the end of June or early July.

Responses for information: Among the NIEHS responses for information prepared, there were those concerned with potential for carcinogenicity of chlordane and haloethers as a potential contaminant of drinking water. Letters concerning a suspected case of DDT poisoning in a human and the possible causes of

toxicity in a horse that ate plastic were routed for further information then answered.

Review of documents and manuscripts: Documents from NIOSH and EPA were reviewed as well as an Environmental Impact Statement from DOT. A major lead paper that appeared in Science was reviewed for DHEW prior to publication.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The project identifies areas where research is needed in order to prevent or reduce the toxicologic hazard from environmental chemicals or from sources of physical stress (the latter as in the IMOS Task Force considerations). It is primarily a program of preventive and early-warning surveillance. It is linked closely to research dealing with the scope of the problem, mechanisms involved and preventive measures. It also takes into consideration potential means of diagnosis and therapeutics where these might be possible.

PROPOSED COURSE: The new findings of biologic and toxicologic importance for the aminimide compounds will be followed as they appear. The vinyl halide situation will be followed closely and the manuscript in preparation will be completed. Additional channels will be sought for appropriate consideration of the hazard of methanol so that, if methanol is to be used in some of the proposed situations, its hazard can be minimized. Presently, it is felt that methanol should not be used in some of the proposed situations. Qualitative aspects of structure-activity relationships will be considered further. Meeting attendance, participation on further projects of the IMOS Task Force, responses for information and review of documents and manuscripts will all be continued.

KEYWORD DESCRIPTORS: preventive surveillance; aminimides; vinyl hylides; methanol; structure-activity; IMOS; ultraviolet; aspartame; fiberglass.

PUBLICATIONS

Bus, J.S., Preache, M.M., Cagen, S.Z., Posner, H.S., Eliason, B.C., Sharp, C.W. and Gibson, J.E.: Fetal toxicity and distribution of paraquat and diquat in mice and rats. Toxicol. Appl. Pharmacol. (in press).

Posner, H.S.: Prenatal maldevelopment. In Principles and Practice of Industrial Toxicology. (in press).

Posner, H.S.: Biohazards of methanol in proposed new uses. J. Toxicol. Environ. Health (in press).

1. Associate Director for Program
- 2.
3. Research Triangle Park, N.C.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

PROJECT TITLE: "Behavioral Tests as Sensitive Indicators of Toxicity"

PREVIOUS PROJECT NUMBER: None

PRINCIPAL INVESTIGATOR: Terri Damstra, Ph.D.

OTHER INVESTIGATORS: Hans L. Falk, Ph.D.

COOPERATING UNITS: None

MAN YEARS

Total:	0.2
Professional:	0.2
Other:	0.0

PROJECT DESCRIPTION

OBJECTIVES: To provide background information about the sensitivity, reliability, and practicality of using behavioral tests as indicators of the potential toxicity of environmental chemical agents. Information on the availability of animal test systems which will reveal behavioral toxicity, and systems for evaluating and monitoring behavioral effects in exposed humans is necessary in order to plan more comprehensive toxicological investigations which might detect functional changes before chemical and morphological changes are apparent.

METHODS EMPLOYED: Literature review to integrate the available information on behavioral toxicity, and a continual literature survey to keep up with developing technologies and discoveries. Consultations and discussions with expert scientists from other Government agencies, industry, and academia.

MAJOR FINDINGS: A large number of industrial and environmental chemicals are reported to affect the central nervous system at the threshold concentration or at concentrations two or tenfold above the recommended level. Apart from direct neurotoxic effects, the perception of toxic environmental stimuli by the central nervous system may affect many other bodily processes; resulting in disturbances in perception, cognition, decision-making, motor activity, vigilance, emotionality, etc. In many instances toxic effects of low concentrations of chemicals can be detected by neurophysiological and behavioral

tests before any morphological, clinical, or biochemical changes are apparent. Thus behavioral tests may serve as sensitive indicators that a toxic action has occurred.

It is very apparent that there is at present a lack of rational criteria for selecting suitable experimental procedures for both animal test systems and monitoring human populations.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The literature surveillance and liaison activities with outside scientists will provide information for the Institute about the applicability and relevance of using behavioral and neurophysiological tests to determine exposure levels for environmental chemicals.

PROPOSED COURSE: This work will continue and expand into the investigation of whether exposure to environmental chemicals during critical periods in fetal development may have subsequent permanent behavioral effects.

KEYWORD DESCRIPTORS: Behavior, Neurophysiology, Central Nervous System, Neurotoxicity.

ENVIRONMENTAL BIOLOGY AND CHEMISTRY BRANCH

ENVIRONMENTAL BIOLOGY AND CHEMISTRY BRANCH

Summary Statement

The Environmental Biology and Chemistry Branch's mission objectives are three fold: 1) maintain collaborative support laboratories in the disciplines of chemistry, microbiology, pathology, histology and electron microscopy, and veterinary medicine; 2) program and coordinate experimental animal procurement, housing and utilization within the Institute; 3) plan and conduct research appropriate to the capabilities of Branch laboratories. Administratively, the Environmental Biology and Chemistry Branch is organized into three Sections: Chemistry, Comparative Biology and Animal Husbandry. Operationally, the utilization of rigid Section structure to achieve Branch objectives is not fostered. Specific work units, the size and composition of each being determined by the task requirements, often draw personnel from two or three Sections. Narratives of activities are presented for work units where appropriate as they portray these efforts in a more comprehensible format.

Support Activities

Chemists are frequently consulted within NIEHS in order to advise scientists in appropriate areas of their research. The support activities of the Chemistry Section fluctuate dependent upon the resources required to meet a particular need. In this respect, the Section is striving to fully develop both analytical and synthetic resources for both labeled and unlabeled compounds to support diverse intramural research programs. The number of chemical service requests are progressively increasing. There is a need to establish some guidelines for chemical services which will nurture well conceived chemical service requests. This is especially needed for synthetic services since many requests involve research synthesis which can require considerable expenditures of time and effort. There is also a clear need for a chemical service for routine chemical disposal of hazardous materials, but this cannot be met by our present staff. The Section also needs to extend its capabilities in the area of inorganics analysis to support diverse studies in bioinorganic chemistry with emphasis on heavy metals. In addition, the Section should begin to explore electrochemistry for determining both inorganics and organics in biological samples.

A major reevaluation of animal support activities commenced during the past fiscal year and is expected to continue into FY 1976. The sometimes harsh realities of experience combined with changing program needs clearly indicated a need for this reevaluation. The finite resources of the animal support programs in terms of space, personnel, quality control laboratories and dollars demand creation of a formal policy defining what animal stocks and services are to be provided in some degree of priority ranking. A formula for allocating space is also needed. To this end, we plan to restrict the number of animal

models available at NIEHS and develop resident research colonies of inbred mice. Restricting the animal models available for general research use to specific species and strains from specific sources will allow development of effective transportation modes, reduce costs, better utilize animal rooms and result in a more effective quality control program through more frequent and extensive evaluation.

The need for development of resident inbred mouse colonies is necessitated by the repeated failure to receive adequate quantities of disease-free mice in a timely fashion from either commercial or other governmental sources. It is proposed that three inbred mice will be available for general NIEHS use. These strains, C57Bl/6, DBA/2 and C3H, will each originate from one genetic foundation stock. These strains will be cesarean derived, given a stable bacterial flora, expanded in number through one generation, and supplied to NIEHS. Population expansion through an additional generation will be effected in rigidly controlled conditions with resultant progeny available for intramural research. Systematic repopulation of the NIEHS colony will produce inbred mice that are not sublined, reliably disease-free through management control of the cesarean derivation process, and physiologically stable by virtue of conception birth and development within the NIEHS environment.

The Health Surveillance Program, composed of personnel from the Animal Husbandry and Comparative Biology Sections, was thoroughly reviewed and revised. In spite of more frequent and early evaluation of animal shipments, the identification of mice and rats infected with mites, or pin worms, exemplify the need for a more comprehensive and effective approach to this problem. It once again reaffirms the variability of shipments from outside sources and the repeated failure of these sources to identify these infestations within their own colonies or communicate these observations to NIEHS prior to being identified by our laboratories.

Research requirements on the part of a number of scientists led to the formation of a rodent breeding and reproduction technical unit within the Animal Husbandry Section. This unit now produces desired quantities of dated pregnant mice and rats.

Training continues to be a major effort in the Animal Husbandry Section. In addition to didactic training, the recently developed AALAS aututorial program was purchased and made available to all employees. Several employees are also taking formal technician training courses offered by the Research Triangle AALAS organization.

Analytical biochemistry and metabolism and organic chemistry and synthesis are areas that rely heavily on specialty instrumentation such as gas chromatography - infrared spectroscopy, high pressure liquid chromatography, and a variety of instruments for detecting and measuring radioactivity.

The more involved organic syntheses have included the cofactor desoxy - nicotinamide-adenine dinucleotide (NAD^+), 3,4,5,3',4',5'-hexachlorobiphenyl in pure form and hydroxylated derivatives, and phenanthrene oxide.

The mass spectrometry (MS) center is continually developing methodology for

qualitative analytical application to biological samples which contain residue levels of environmental agents. Of particular interest is the use of selective reagent gases in chemical ionization mass spectrometry. Preliminary work indicates that diacetyl may be a suitable reagent for this purpose. MS is generally useful for determining the "chemical appearances" of environmental agents for possible correlation with biological activity.

The nuclear magnetic resonance (NMR) facility is likewise developing methodology for qualitative application to biological samples as well as chemical reaction products. Preliminary studies with ^{13}C -enriched benzene has indicated that NMR may be useful in at least qualitatively determining metabolism via analyses of body fluids and tissues or their crude extracts and homogenates. The value of ^{13}C -enriched compounds in elucidating mechanisms of lipophilicity and carcinogenicity is being investigated.

High pressure liquid chromatography (HPLC) is being increasingly used to support diverse metabolism, chemical purity, and other studies. It was particularly useful in examining the purity of some progesterone steroid derivatives and for measuring partition coefficients of hexachlorobiphenyl isomers.

Research Activities

There are four major research involvements of the Chemistry Section which are using specific classes of chemical compounds as models to develop general methodology of value to environmental health scientists.

1. One involvement concerns the development of a radioimmunoassay method for 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) which will be both highly sensitive and specific. Suitable haptens of TCDD (1-amino and its succinic acid amide derivative) have been prepared. Using a model compound (4-amino-4'-chlorobiphenyl), two methods of hapten protein covalent bonding (antigen) have been examined, viz the carbodiimide and diazo reactions. The diazo method was successful in preparing an antigen of 1-amino TCDD which remains to be fully characterized. A second model (2,3,5,6-tetrachloroaniline) is being used to develop the N-hydroxy succinimide activated ester method for antigen preparation. Preliminary immunology has begun with the diazo type antigen and its reduced form (hydrazine linkage) in collaboration with the Comparative Biology Section.

2. Hexachlorinated biphenyl studies are aimed at correlating the physical-chemical properties of polychlorinated biphenyls with their toxicological properties. The biological activity of five symmetrical hexachlorobiphenyl isomers was shown to correlate reasonably well with their retention indices as measured by gas chromatography. This activity is more directly correlatable with their lipophilicity as measured by tissue uptake. Physical-chemical data as measured spectroscopically supported the importance of 3 (and 5) and 4 substitution of chlorine for high polarity and lipophilicity. More recent studies have indicated the 3,4,5,3',4',5'-isomer to be highly active at 100 ppm while a hydroxylated derivative (2-OH) was relatively inactive at 10 and 30 ppm. These studies permit differentiation between a dibenzofuran and a PCB effect.

3. Studies are underway on the capabilities of various tissue lipases and esterases as early mediators in the interaction between mammals and xenobiotics containing ester linkages. It is of particular interest to understand how lipases are able to bind to and react with water-insoluble compounds such as the herbicides, 2,4-D and 2,4,5-T, phthalate plasticizers, carbamate insecticides, etc. and also if this hydrolytic process is always detoxifying. Rat lung, skin, and pancreas homogenates show distinctive patterns of hydrolytic activity. A procedure for purifying the micelle lipase from rat pancreas has been developed, and characterization of the lipase is in progress. The mechanism is being studied by ^{18}O -enriched water experiments. The apparent K_m is independent of the nature of the ester substrate, and is dependent only on the type of bile salt used to form micelles. V_{max} was dependent upon the substrate.

4. The biomimetic ability of certain well defined (mechanistically) chemical reactions is being studied. The chlorinated polycyclodiene pesticides are being used as models to study the chlorination process. Dechlorination can occur environmentally under a variety of conditions, viz photochemically, chemically, biologically, and under gamma irradiating conditions. Some of these conditions have been shown to accomplish regiospecific and stereoselective reductive dechlorination as well as lead to partial molecular skeletal rearrangement. Whether or not these processes are detoxifying in view of the possible formation of compounds related to vinyl chloride and chlorinated aromatics is being considered.

Many of the specific research projects within the Comparative Biology Section interrelate to compose a larger program that focuses on the effect of environmental agents on host resistance. This program hopes to define test methods that are most appropriate for detecting effects as well as identify which biological systems are involved.

The cellular and humoral components of the immune system are a major area of study. Endocrine function is also studied to allow a distinction between direct immune effects and immune alterations that are secondary to endocrine change.

The consequences of exposure to environmental chemicals during immune ontogenesis continues to be studied. Is the immune system more subject to deleterious effects during development? Are immunosuppressive effects of a transient or permanent nature? Previous Branch research has shown the chemical TCDD to be immunosuppressive. Current experiments suggest that the TCDD induced T-cell suppression may be transient. This cell mediated suppression may also be selective since the hypersensitivity response to oxazalone remained severely depressed. The nature and temporal consequence of these effects continue to be investigated.

Another area of research evaluates host resistance by testing the ability of mice to withstand challenge exposure to bacteria or virus. Mice treated with small doses of TCDD (one tenth the LD_{50}) were found to have greater susceptibility to subsequent bacterial challenge as measured by the proportion of mice that die or decreased survival time. This effect is not felt to be related to

an impaired immune system. The status of reticuloendothelial system and phagocytic capabilities of circulating leukocytes are currently being studied.

Another model that seeks to assess the interaction of environmental compounds and development of disease is the induction of tumors by avian sarcoma virus (ASV). Refinement of the model continued with particular focus on the correlation of age at time of dosing and tumor incidence or time to tumor development. Hemorrhagic cysts, often at the site of superficial lymph nodes are seen in rats inoculated with ASV as neonates. Studies with rats with these cysts find them to be immuno-depressed.

Investigations into the patterns and nature of chlorinated dibenzodioxin toxicity continued. A study of selected dioxin isomers commenced using mice and guinea pigs. The single oral LD₅₀ dose and characterization of the toxic response primarily by histopathologic study are being determined. With these data we hope to evaluate the hypothesis of Poland and Glover that relates degree and location of halogens to toxicity.

A series of systematic experiments are being conducted to collate the interactive effects of TCDD on various physiologic functions as these changes may contribute to thymic involution. Results to date indicate that the thymus atrophy is not secondary to adrenal or pituitary (somatotrophic hormone) dysfunction. Reduced caloric intake, which is observed following TCDD dosing, is not in itself the prime cause of thymic atrophy.

Branch personnel are collaborating with the Veterinary Resources Branch, DRS, in attempting to characterize and define the cause of a fatal wasting disease in Macaca mulatta monkeys. The similarity of the disease process to that produced by chlorinated cyclic hydrocarbons such as polychlorinated biphenyl or dibenzodioxin is quite striking. Attempts to isolate and identify these chemicals in tissues from affected monkeys have failed to yield them in quantities that allow one to implicate them as a cause of the disease.

Safety Programs

This Branch is actively committed to implementation of the recently developed NIEHS Safety Program. Much of the daily routine necessitates the use of toxic materials. All non-work related activities are directed to clean areas that have been set aside for these purposes. Specific procedures for chemical disposal of various classes of hazardous compounds are being developed.

Project No.: Z01 ES 30001-04 EBCB

1. Environmental Biology and Chemistry Branch
2. Comparative Biology Section
3. Research Triangle Park, N.C.

PHS-NIH

Individual Project Report

July 1, 1974 through June 30, 1975

PROJECT TITLE: "Health Surveillance of Laboratory Animals at NIEHS"

PREVIOUS SERIAL NUMBER: NIEHS-EBC-001-3

PRINCIPAL INVESTIGATOR: Donald B. Feldman, D.V.M.

OTHER INVESTIGATORS: Bhola N. Gupta, B.V.Sc.&A.H., Ph.D., Julius E. Thigpen, Ph.D.

COOPERATING UNITS: Animal Husbandry Section, EBCB

MAN YEARS

Total:	3.5
Professional:	1.2
Other:	2.3

PROJECT DESCRIPTION

OBJECTIVES: To monitor incoming and resident laboratory animals for spontaneous infectious diseases and parasitism.

METHODS EMPLOYED: All research animals at NIEHS are routinely observed for clinical signs of disease. Animals to be selected for health surveillance are chosen one week in advance at a weekly conference. Any problems relating to animal disease or husbandry may be discussed at this meeting.

New species or strains of animals from new sources undergo complete monitoring, including examination for respiratory and enteric pathogens, ecto- and endoparasites and incidental pathology. Reference histological slides are kept on each strain or species. Pooled sera are to be periodically screened for viral serology.

Resident animals are monitored approximately every 6 weeks for respiratory pathogen and parasites. Only tissues from major organs are collected in these cases.

Moribund or dead animals on experiment are referred to the investigator to determine if the condition is spontaneous or experimentally induced. Dead, diseased or abnormal animals are usually submitted to necropsy, after clinical examination.

MAJOR FINDINGS: Incoming and resident rats, monitored at monthly intervals have been free of salmonellosis. Chronic respiratory disease (CRD) due to Mycoplasma pulmonis (PPL0) has become endemic in CD rats but the incidence of overt clinical disease is low. Pasteurella pneumotropica is occasionally cultured from the lungs of clinically normal animals. Murine pinworm (Syphacia sp.) was detected in resident Fisher 344 rats but animals have been parasite free for the past 6 months. Recently, Syphacia sp. was detected in the feces of a newly arrived CD rat. Murine pinworm is innocuous and species related but the presence of this parasite is esthetically displeasing. Treatment is usually futile. Attempts are being made to isolate infected animals and to assure that new arrivals are parasite-free.

Swiss and inbred mice have been relatively free of CRD. Pinworm infection has spread among mice after initial contamination by CD-1 mice. A number of C3H/Hen mice were found with inner ear infections (labyrinthitis) due to Pseudomonas aeruginosa. This colony was eliminated. A "bloat syndrome" has been detected in C57Bl/J67) (SPF) mice, especially among males 12-24 hours following mating. Females are less often affected. The entire gastrointestinal tract is extremely dilated with gas or air and suffocation results from pressure on the diaphragm. Aerobic and anaerobic microbiological cultures have been unrevealing.

Vertical pillars or septa at the entrance to the vagina have been found in some breeding C57Bl/J67 female mice. The incidence of this congenital anomaly has been calculated to be approximately 1%. This condition has not been reported heretofore but mouse breeders occasionally see this anomaly (personal communications). Recently similar defects were found in CD-1 mice at NIEHS.

Guinea pigs and hamsters are purchased from reliable commercial sources and have been relatively disease free.

The incidence of "snuffles" (pasteurellosis) in NZW rabbits has not changed considerably (approximately 30%). Mucoïd enteritis is now rarely observed in large female NZW rabbits whereas the incidence is higher in male NZW (SPF) from another source.

Fatalities among breeding opossums are relatively high. Deaths result from septicemia as an aftermath of fighting.

The following cases are worth mentioning, and case reports are in preparation for publication in professional journals.

1. Mycobacterial septicemia in an opossum - acid-fast bacilli were observed microscopically in the spleen, liver, lung, kidney and pancreatic lymph node. Mycobacterium avian complex - serotype 6 was isolated.
2. Peliosis hepatis in the opossum: Liver changes in 3 opossums were characterized by blood containing cystic spaces into the parenchymatous tissue.
3. Lipoma in an opossum. A small pedunculated mass on the elbow of an adult captive born opossum. The neoplasm was composed primarily of lipocytes.
4. Neurofibroma in an opossum. A neoplastic growth of mesenchymal origin was

observed in the retrobulbar area of a female captive born opossum.

5. Extramural thrombosis in a guinea pig. Small "millet-like" bodies were observed in the pericardial sac which had the microscopic appearance of healed thrombi. Diplococcus pneumoniae was isolated.

6. Trichoepithelioma in a guinea pig. A subcutaneous nodule composed of adnexal component was observed in a 9-month-old male guinea pig.

7. Intrathoracic dermoid cysts in a rat. Several dermoid cysts were observed on the ventral surface of the thoracic cavity of a 6-month-old female rat (Buffalo strain).

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The health status of all research animals must be continually monitored; otherwise, intercurrent infections could cause misinterpretation and possible negation of valuable experimental data. In addition, microbiological and histopathological surveillance has uncovered interesting and valuable data heretofore unpublished in laboratory animal medicine.

PROPOSED COURSE: Surveillance of laboratory animals will continue on a routine basis.

KEYWORD DESCRIPTORS: Diseases of laboratory animals; animal husbandry; animal health surveillance.

PUBLICATIONS

Gupta, B.N. and Feldman, D.B.: Renal agenesis in the guinea pig and opossum. Lab. Anim. Sci. 25: 238-240, 1975.

Gupta, B.N.: Duplication of gall bladder in a rabbit. Lab. Anim. Sci. (Accepted), 1975.

Gupta, B.N.: Ectopic kidney in a rat. Lab. Anim. Sci. (Accepted), 1975.

Gupta, B.N. and Dennis, R.: Tissue washing tank for histologic preparations. Am. J. Vet. Res. (Accepted), 1975.

Project No.: Z01 ES 30002-02 EBCB

1. Environmental Biology and Chemistry Branch
2. Chemistry Section
3. Research Triangle Park, N.C.

PHS-NIH

Individual Project Report

July 1, 1974 through June 30, 1975

PROJECT TITLE: "Organic Synthesis of Environmental Agents and Metabolites (Labeled and Unlabeled) and Development of Organo-Analytical Methodology"

PREVIOUS SERIAL NUMBER: NIEHS-EBC-002

PRINCIPAL INVESTIGATOR: J. D. McKinney, Ph.D.

OTHER INVESTIGATORS: M. Walker, L. Levy, Ph.D., K. Chae, Ph.D., R. Thomas
and J. Fawkes

COOPERATING UNITS: Institute wide

MAN YEARS

Total:	1.0
Professional:	0.4
Other:	0.6

PROJECT DESCRIPTION

OBJECTIVES: To provide intra- and interbranch synthetic and radio-synthetic capabilities as well as develop organo-analytical methodology as required to support diverse biological studies with environmental agents and their transformation products.

METHODS EMPLOYED: A variety of synthetic, chromatographic, autoradiographic, and spectroscopic techniques including radio-GC and TLC, high pressure liquid chromatography, GC-IR, UV, liquid scintillation counting, etc.

MAJOR FINDINGS: This report covers chemical services of an organic or organo-analytical nature only. Biochemical services and services involving specialty instrumentation are covered by separate reports.

Since the institute radiochemical contract has been in force, the service load for our radiosynthetic laboratory has been reduced primarily to end-of-sequence labeling which is more tenable with our existing facility. The labeled syntheses include ^{14}C -syn-dechlorodieldrin from dieldrin, a 15-keto- $[\text{}^3\text{H}]$ prostaglandin derivative from $\text{F}_{2\alpha}\text{-9-}^3\text{H}$, the disodium salt of ^{14}C -ethylene diamine tetraacetate from free acid, and the ^{14}C -monomethyl ester and monoacid chloride of succinic acid from succinic anhydride.

Unlabeled synthesis are more frequently requested and the more difficult include desoxy NAD⁺, 3,4,5,3',4',5'-hexachlorobiphenyl in pure form and hydroxylated derivatives, and phenanthrene oxide.

Quite often chemical purity checks are requested and include thalidomide, metronidazole, some progesterone derivatives, and samples of dipterex and imidan (in cooperation with Soviet scientists).

In regard to affects on biological test systems, the institute tap water supply is being analyzed in hopes of identifying the causal factors. Several approaches are being taken to identify both the dissolved organics and inorganics.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Radio-labeled compounds are particularly desirable biological tracers for facilitating diverse biological studies with environmental agents. In addition, new and improved analytical methodology is continually required to enable unequivocal identification with better sensitivity (minimum detectability).

PROPOSED COURSE: To continue to develop new and improved synthetic, radio-synthetic, organo-analytical methodology for preparing and analyzing environmental agents and their transformation products.

KEYWORD DESCRIPTORS: Synthetic services; organo-analytical methodology; purity analysis.

Project No.: Z01 ES 30003-03 EBCB

1. Environmental Biology and Chemistry Branch
2. Chemistry Section
3. Research Triangle Park, N.C.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

PROJECT TITLE: "Development of Analytical Methodology"

PREVIOUS SERIAL NUMBER: NIEHS-EBC-003-2

PRINCIPAL INVESTIGATOR: P. W. Albro, Ph.D.

OTHER INVESTIGATORS: R. Thomas, J. Fawkes, B. J. Corbett

COOPERATING UNITS:

MAN YEARS

Total:	1.9
Professional:	0.1
Other:	1.8

PROJECT DESCRIPTION

OBJECTIVES: To develop and refine methodology for the quantitative and qualitative determination of compounds and classes of compounds of general interest to the Institute and specific interest to individual investigators. In general, compounds covered by this study will include (1) those for which there is no analytical methodology, insufficiently specific or sensitive methodology, or methodology we are not equipped to utilize. (2) those which are metabolites of compounds for which adequate analytical methodology is available, and (3) those for which an adequate analytical procedure is available but which occur in a milieu providing interference with the standard procedure.

METHODS EMPLOYED: The analytic methods will generally be developed using gas chromatography, thin-layer and column chromatography, fluorometry, spectrophotometry (IR, UR, Visible), mass spectrometry and isotopic methods. Other special methods will be employed where necessary or as other instrumentation becomes available.

MAJOR FINDINGS: Attempts to analyze brain extracts for amphetamine metabolites have resulted in the development of a successful derivatization procedure. However, attempts to produce electron-capturing derivatives for GLC have been frustrated by the extremely "dirty" reagents commercially available. The most promising approach at the moment appears to be formation of O-trimethylsilyl, N-heptafluorobutryl derivatives, and this is presently under investigation.

Ten methods of protein assay were compared using insoluble (particulate) protein preparations as samples. The standard Lowry procedure was invalid when applied to calcium-precipitated microsomes. Detailed results were accepted for publication: [Albro, P.W., Anal. Biochem. (in press)].

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Successful development of the analytical methodology in the areas delineated above will accelerate the successful elaboration of a number of metabolic and degradation studies in progress in the Chemistry Section as well as be useful for other studies within and without the Institute.

PROPOSED COURSE: We will continue to attempt to develop analytical methodology as required under the terms of Objectives above. In particular, an attempt will be made to develop a radioimmunoassay for tetrachlorodibenzodioxins based upon derivatives other than the amine.

KEYWORD DESCRIPTORS: Analytical methodology; tetrachlorodibenzodioxins; amphetamines; protein assays; gas chromatography; fluorometry; spectroscopy; isotopic methods.

PUBLICATIONS

Oswald, E.O., Albro, P.W. and McKinney, J.D.: Utilization of gas-liquid chromatography coupled with chemical ionization and electron impact mass spectrometry for the investigation of potentially hazardous environmental agents and their metabolites. J. Chromatogr. 98: 363-448, 1974.

Project No.: Z01 ES 30004-03 EBCB

1. Environmental Biology and Chemistry Branch
2. Chemistry Section
3. Research Triangle Park, N.C.

PHS-NIH

Individual Project Report

July 1, 1974 through June 30, 1975

PROJECT TITLE: "Analytical Services: Mass Spectrometry and Gas Chromatography-Mass Spectrometry"

PREVIOUS SERIAL NUMBER: NIEHS-EBC-004-2

PRINCIPAL INVESTIGATOR: J. Ronald Hass, Ph.D.

OTHER INVESTIGATORS: Mr. R. O. Thomas

COOPERATING UNITS: None

MAN YEARS

Total:	0.4
Professional:	0.3
Other:	0.1

PROJECT DESCRIPTION

OBJECTIVES: The purpose of this project is to provide a mass spectrometry, and combined gas chromatography-mass spectrometry service for the intramural research program at NIEHS. It is intended that this service should be useful to investigators with no expertise in either GC or MS.

METHODS EMPLOYED: The existing GC/MS-computer equipment at NIEHS was used in the organization of a GC/MS service facility. Services available include combined GC/MS with either electron impact or chemical ionization and automated data acquisition via computer control of the mass spectrometer. Samples of reasonably pure solids can be introduced into the ion source by means of a direct insertion probe. Necessary GC methods development is accomplished by means of independent gas chromatographs.

MAJOR FINDINGS: During the period August 15, 1974 - March 31, 1975, the NIEHS Mass Spectrometry Center responded to 58 requests for service which included a total of 80 samples. Of these, 39 were concerned with metabolite identification. The majority of the remainder were analyzed in support of various organic synthesis projects within the Chemistry Section and the identification of substances in environmental samples. Of the 80 samples, 58 required GC/MS analysis and 22 were analyzed by means of the direct probe inlet.

In addition to the above service a major analytical service project has involved attempts to identify the factor leading to the deaths of monkeys in the Primate

Center at Perrine, Florida. Chemical analysis of monkey tissue samples indicated the presence of 2-5 ppm PCBs in the fat of one of the diseased monkeys. Attempts at the identification of other possible agents have suffered from high background levels of biological chemicals in the extract samples submitted for analysis and relatively poor sensitivity on the part of the mass spectrometer. This project is being pursued by doing further clean-up of the samples and upgrading the mass spectrometer to latest operating specifications.

A sub-project of the mass spectrometry center has been the reorganization of the mass spectral data acquired from 1971-1974. Much of these data were stored on magnetic tape in a format that cannot be decoded by the disk operating system of the computer. These data have been converted to the proper format for use with the disk system. This will allow much faster data manipulation by the computer. The next stage will involve the evaluation of these data and the eventual development of a computer searchable mass spectral library. This will speed interpretative analytical services by allowing computer matching of unknown mass spectra to mass spectra of compounds that have been studied previously.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: This project provides a basic analytical service necessary for many of the intramural research programs at NIEHS. The application of combined GC/MS to problems in biomedical research is a rapidly growing area of analytical chemistry. The provision of this capability to investigators without the necessity of their developing expertise in the area should lead to greater efficiency in the intramural research program.-

PROPOSED COURSE: To be continued.

KEYWORD DESCRIPTORS: Mass Spectrometry; GC/MS; Analytical Services.

Project No.: Z01 ES 30005-06 EBCB

1. Environmental Biology and Chemistry Branch
2. Comparative Biology Section
3. Research Triangle Park, N.C.

PHS-NIH

Individual Project Report

July 1, 1974 through June 30, 1975

PROJECT TITLE: "Toxicity of Dibenzodioxins"

PREVIOUS SERIAL NUMBER: NIEHS-EBC-005-5

PRINCIPAL INVESTIGATOR: John A. Moore, D.V.M.

OTHER INVESTIGATORS: Ernest E. McConnell, D.V.M., Bhola N. Gupta, B.V.Sc.&A.H.,
Ph.D., and Martha Harris

COOPERATING UNITS: J. D. McKinney, Ph.D., Chemistry Section, EBCB

MAN YEARS

Total:	2.3
Professional:	0.8
Other:	1.5

PROJECT DESCRIPTION

OBJECTIVES: Using pure chlorinated dibenzodioxins, determine the dose required to produce a toxic or lethal response in laboratory animals. Identify the sequence of toxicopathological processes associated with selected doses of compound. Investigate if interactions occur in vivo with the dioxin isomers i.e. are there additive, antagonistic or synergistic effects on toxicity.

METHODS EMPLOYED: Mice, and guinea pigs were given single or multiple doses of various dibenzodioxins (see below) orally in order to study the toxicologic, pathologic and clinical pathologic effects of the intoxication. Standard techniques were used in order to evaluate these effects.

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD), 1-NH₂-2,3,7,8 TCDD, 1-NO₂-2,3,7,8 TCDD, 1,2,3,4,7,8-hexachlorodibenzo-p-dioxin (HCDD) and 1,2,3,6,7,8-HCDD.

MAJOR FINDINGS: The toxicopathology of the above compounds was studied in C57B1/6gh (J67) mice and Hartley guinea pigs. The single oral LD₅₀ was determined for these compounds in both species of animals. 2,3,7,8-TCDD was found more toxic than any of the other isomers. The lesions produced by all compounds were similar in a given species of animal. However, a distinct difference was noted between species. Thymic and splenic atrophy, toxic hepatitis, vascular lesions (edema and hemorrhage) and serum protein dyscrasias were the prominent features in mice. In contrast, thymic, testicular and bone marrow atrophy along with adrenal cortical hemorrhage and proliferation of the

renal pelvic epithelium were more characteristically found in guinea pigs.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Chlorinated dibenzodioxins may occur in association with many useful chlorinated cyclic compounds. The symmetrical tetrachloro isomer is one of the most toxic chemicals known. The pattern of toxicity associated with most of the isomers has not been determined. The interactions of isomers as they relate to toxicity is unknown. These facts must be determined if assessment of the environmental health implication of products which contain dibenzodioxin isomers as contaminants are to be made.

PROPOSED COURSE: To be continued.

KEYWORD DESCRIPTORS: Dibenzodioxins; toxicity; additive, antagonistic or synergistic effects.

PUBLICATIONS

Vos, J.G., Moore, J.A., and Zinkl, J.G.: Toxicology of 2,3,7,8-tetrachloro-dibenzo-p-dioxin (TCDD) in C57Bl/6 mice. Toxicology and Applied Pharmacology 29: 229-241, 1974.

Project No.: Z01 ES 30006-01 EBCB

1. Environmental Biology and Chemistry Branch
2. Comparative Biology Section
3. Research Triangle Park, N.C.

PHS-NIH

Individual Project Report

July 1, 1974 through June 30, 1975

PROJECT TITLE: "Mode of Action of 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD)"

PREVIOUS SERIAL NUMBER: None

PRINCIPAL INVESTIGATOR: M. J. van Logten, Ph.D.

OTHER INVESTIGATORS: Bhola N. Gupta, B.V.Sc.& A.H., Ph.D. and John A. Moore, D.V.M.

COOPERATING UNITS: None

MAN YEARS

Total:	1.6
Professional:	1.1
Other:	0.5

PROJECT DESCRIPTION

OBJECTIVES: To determine the mode of action of TCDD, especially with respect to the influence on the thymus, in order to evaluate the possible hazards of immune suppression for man and animal.

METHODS EMPLOYED: Normal rats and guinea pigs were given single oral doses of TCDD. The animals were killed either after 3, 10 or 21 days. Some experiments were carried out with adrenalectomized or hypophysectomized rats. In guinea pigs Metyrapone was injected in order to mimic adrenalectomy. To test the hypothesis that TCDD may block the STH receptor sites in the thymus, normal and hypophysectomized rats were injected with growth hormone prior to TCDD administration. In a few experiments caloric deprivation was used to correct for the decrease in food intake after TCDD dosing. In the thymus of the rats, DNA, RNA and protein determination were carried out. Experiments to study the influence of TCDD on the DNA-synthesis are underway. To look for a relation between the wasting syndrome after TCDD dosing and protein deficiency an experiment is going on with several protein levels in the diet.

MAJOR FINDINGS: 1. The effects of TCDD on the thymus of both adrenalectomized animals and normal animals seems to be the same. This tends to rule out that alterations in the thymus are secondary to hyperadrenal activity.

2. Hypophysectomy in rats causes a mild atrophy of the thymus. It is unlikely that the influence of TCDD on the thymus is mediated by the pituitary gland as

TCDD elicits an additional atrophy of the thymus in hypophysectomized rats.

3. In hypophysectomized rats administration of growth hormone reversed the atrophy of the thymus, whereas thymic atrophy caused by TCDD is not reversed by growth hormone. These results do not support the view that TCDD causes a blockade of the STH receptor sites in the thymus.
4. The observed atrophy of the thymus after a single dose of TCDD is partly due to the reduced food and water intake. However, in caloric deprived animals TCDD induced an additional atrophy.
5. TCDD has no clear effect on the body temperature, the glucose content of the blood or the glycogen concentration of the liver.
6. In rats TCDD causes a dose dependent decrease of the DNA, RNA and protein content of the thymus. A decrease in the concentration of RNA, protein and especially DNA was also observed. These findings support the histological observations that the atrophy of the thymus is due to a decrease in small cortical thymocytes.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: It is important to establish possible specific effects of environmental contaminants on the immune system. It must be determined, if the influence of certain chemicals on the immune system is mediated by the endocrine system and nutrition, or if other factors are involved.

PROPOSED COURSE: To be continued.

KEYWORD DESCRIPTORS: TCDD; thymus; adrenalectomy; hypophysectomy; growth hormone; nutrition; DNA-, RNA- and protein determinations.

Project No.: Z01 ES 30007-03 EBCB

1. Environmental Biology and Chemistry Branch
2. Comparative Biology Section
3. Research Triangle Park, N.C.

PHS-NIH

Individual Project Report

July 1, 1974 through June 30, 1975

PROJECT TITLE: "Effect of Environmental Chemicals on Immunocompetence"

PREVIOUS SERIAL NUMBER: NIEHS-EBC-014-2

PRINCIPAL INVESTIGATOR: Robert E. Faith, D.V.M., M.S.

OTHER INVESTIGATOR: John A. Moore, D.V.M.

COOPERATING UNITS: None

MAN YEARS

Total:	1.5
Professional:	0.7
Other:	0.8

PROJECT DESCRIPTION

OBJECTIVES: To determine the effects, if any, of selected environmental chemicals on the immune competence of laboratory animals and to develop a standard methodology for assessing the effects of environmental chemicals on the immune response.

METHODS EMPLOYED: Cell mediated (T-cell) immunity is being assessed in vitro by the response of thymic or splenic cell cultures to the mitogens Concanavalin A or phytohemagglutinin and in vivo by delayed hypersensitivity response to the contact sensitizing agent 4-ethoxymethylene-2-phenyl-2-oxazolone (oxazolone). The use of graft versus host reactions and allograft rejection has been discontinued due to the difficulty in quantitating these reactions. Humoral immunity (B-cell mediated) is being assessed by the response of splenic cell cultures to the mitogens E. coli 055 B5 lipopolysaccharide and dextran sulfate (B-cell mitogens) and by passive hemagglutination or radial diffusion to measure antibody responses to both T-independent and T-dependent antigens. In addition to immunologic parameters animals are observed for signs of general toxicity.

MAJOR FINDINGS: Current studies are investigating the effects of maternal exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) or methyl mercury on the immune competence of the juvenile offspring. The earlier findings of Vos and Moore have been confirmed and the effects of TCDD on the developing immune system are being further investigated. While TCDD initially depresses the in vitro response of T-cells to mitogenic stimulation this response tends to

return towards normal as time post exposure increases. However, even when the response of T-cells to mitogenic stimulation has returned to close to normal the animals delayed hypersensitivity response to oxazolone is still severely depressed. Initial findings indicate that TCDD has no effect on humoral immunity suggesting that TCDD selectively suppresses T-cell mediated immunity. Initial studies with methyl mercury indicate that maternal exposure to this compound suppresses humoral immune responses while not significantly effecting cell mediated immunity. Methyl mercury will induce immune suppression at levels lower than those required for induction of overt teratology.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Environmental contaminants may exert subtle effects on man and animals. Among these effects may be a modulation of immune response, either suppression or enhancement. It is important to establish which environmental chemicals may induce modulation of immune responses, and their mode of action, in order to evaluate the hazards of immune modulation for men and animals.

PROPOSED COURSE: To be continued.

KEYWORD DESCRIPTORS: Environmental chemicals; immunocompetence; delayed hypersensitivity; mitogen response; immune suppression; 2,3,7,8-tetrachlorodibenzo-p-dioxin; methyl mercury.

PUBLICATIONS

Vos, J.G. and Moore, J.A.: Suppression of Cellular Immunity in Rats and Mice by Maternal Treatment with 2,3,7,8-Tetrachlorodibenzo-p-Dioxin. Int. Arch. Allergy Appl. Immunology 47: 777-794, 1974.

Project No.: Z01 ES 30008-03 EBCB
1. Environmental Biology and Chemistry Branch
2. Comparative Biology Section
3. Research Triangle Park, N.C.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

PROJECT TITLE: "The Effects of Environmental Chemicals on Host Resistance to Infectious Agents"

PREVIOUS SERIAL NUMBER: NIEHS-EBC-017

PRINCIPAL INVESTIGATOR: Julius E. Thigpen, Ph.D.

OTHER INVESTIGATORS: John A. Moore, D.V.M., Ernest E. McConnell, D.V.M. and Robert E. Faith, D.V.M, M.S.

COOPERATING UNITS: Histology; Animal Housing; Biometry (Dr. Haseman)

MAN YEARS

Total:	0.55
Professional:	0.20
Other:	0.35

PROJECT DESCRIPTION

OBJECTIVES: 1. To develop test systems which determine the effects of environmental pollutants on disease resistance of laboratory animals as measured by mortality rates subsequent to exposure to infectious agents. More specifically to determine whether any interaction (antagonistic or synergistic) occurs in dioxin (TCDD) exposed mice when infected with a bacterium (Salmonella bern) or a virus (Pseudorabies-PRV).

METHODS EMPLOYED: Pseudorabies virus will be produced in quantity employing rabbit kidney (RK-13) monolayers and stored in aliquots at -70°C until ready for use. A lethal dose response curve will be established in 8 week-old mice, by inoculating 20 mice/group with 0.2 ml (IP) of varying dilutions of PRV. A LD₂₀ dose will be determined and used as the viral inoculum in the blind study.

Salmonella bern will be grown overnight on blood agar and a bacterial suspension prepared in 0.85% saline. Bacterial concentrations will be determined by the spectrophotometer (% transmittance - 540 mμ) and confirmed by total plate counts (TPC). A lethal dose response curve will be established in C57Bl/6JFH (J67) normal mice by inoculating 0.2 ml (IP) of various dilutions of bacteria. Mortality rates will be determined for 14 days with the bacteria and virus inoculated mice and the dead mice examined by both histologic and microbiologic methods to establish the cause of death.

Experiment-Blind-Study - 3 treatment groups (5, 10, and 20 $\mu\text{g/Kg}$ body wt.) and a control group (60 mice/group randomly distributed) of 5-week-old mice will be dosed each week by gastric intubation for 4 weeks with the appropriate chemical (TCDD and Carrier) and carrier alone (controls). Two days after last treatment (8 weeks-old), the 4 groups of 60 mice/group will be randomly split into 3 groups of 20 mice/group and inoculated with the LD₂₀ dose of Salmonella bern, PRV and saline (diluent). Mortality rates will be recorded daily for 14 days in all groups. Mortality rates in the 3 TCDD exposed groups will be compared to mortality rates in the control. A comparison will also be made between bacteria and virus groups. All data will be analyzed statistically by Dr. Haseman. Three mice from each group will be examined by both histologic and microbiologic methods to establish cause of death.

MAJOR FINDINGS: Lethal dose curves have been established in C57B1/6JFH (J67) mice for both the bacteria (Sal. bern) and virus (PRV). An LD₂₀ dose has been determined for each and the test system results are reproducible as indicated by mortality rates.

Preliminary studies in mice exposed to 5, 10, and 20 μg of TCDD/Kg of body weight for 4 weeks and then infected indicate that (1) TCDD did not increase mortality rates in the treatment groups when compared to the controls in the PRV infected mice; however, (2) TCDD shortens the incubation period and increased mortality rates in the treatment groups over the controls in the Salmonella bern infected group.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: A variety of environmental chemicals when administered in sublethal doses are immunosuppressive and increased mortality rates have been observed upon exposure to infectious agents. These findings are significant in that humans may be exposed to these same compounds in minute quantities and therefore may be rendered more susceptible to certain types of infectious agents. TCDD is immunosuppressive, however, it is not known whether TCDD exposed mice are more susceptible to bacterial or viral infections as determined by mortality rates.

The Salmonella bern lethal dose curve (test system) in normal mice will allow us to define the degree of interrelationship that exist between environmental chemicals (such as TCDD) and increased or decreased susceptibility.

PROPOSED COURSE: Additional studies at 5, 1 and 0.5 $\mu\text{g/Kg}$ with subsequent Salmonella challenge to find "no effect" dose. Assess into leukocyte function as possible factors in bacterial and reticuloendothelial susceptibility.

KEYWORD DESCRIPTORS: (1) Influence of TCDD on mortality rates in mice infected with bacteria or virus. (2) Interaction of TCDD with Sal. bern and PRV. (3) Effects of TCDD on host susceptibility to infectious agents. (4) TCDD increases mortality rates in bacterial infections.

Project No.: Z01 ES 30009-01 EBCB
1. Environmental Biology and Chemistry Branch
2. Chemistry Section
3. Research Triangle Park, N.C.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

PROJECT TITLE: "Chemical Aspects of a Radioimmuno Assay Method for 2,3,7,8-Tetrachlorodibenzo-p-dioxin"

PREVIOUS SERIAL NUMBER: None

PRINCIPAL INVESTIGATOR: J. D. McKinney, Ph.D.

OTHER INVESTIGATORS: P. Albro, Ph.D., K. Chae, Ph.D. and J. Fawkes

COOPERATING UNITS: Comparative Biology Section, EBCB, NIEHS

MAN YEARS

Total:	1.2
Professional:	0.6
Other:	0.6

PROJECT DESCRIPTION

OBJECTIVES: To develop a sensitive and specific radioimmunoassay method for 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD).

METHODS EMPLOYED: A variety of synthetic, chromatographic, and spectroscopic techniques were employed.

MAJOR FINDINGS: The 1-amino and nitro derivatives of TCDD were prepared in pure forms. Silica gel column chromatography was used to separate the mixture of isomeric nitro compounds before reduction to the amino compound. The succinic acid amide derivative of the 1-amino TCDD was also prepared for use as an alternative hapten and remains to be purified.

A model compound, 4-amino-4'-chlorobiphenyl was used in developing methodology for antigen formation from an aromatic amino compound and the proteins, BSA and HSA. Two methods of hapten protein covalent bonding have been examined thus far, viz. the carbodiimide reaction and the diazo reaction. The diazo method was successful in preparing an antigen of 1-amino TCDD which remains to be fully characterized. Dialysis, centrifugation and millipore filtration were used for purification. Using 2,3,5,6-tetrachloroaniline as a model and the succinamide linkage, the chemistry of the N-hydroxy succinimide activated ester method is being explored. This is expected to yield a more definable antigen. Preliminary immunology has begun using the diazo type antigen and its reduced form (hydrazine linkage).

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Chlorinated dibenzodioxins may occur in association with many extensively used chlorinated aromatic compounds. The 2,3,7,8-tetrachloro isomer is one of the most toxic chemicals known. A sensitive and specific analytical method which is both economic and convenient is needed.

PROPOSED COURSE: The reduced form of the diazo type antigen will be further investigated as a more stable antigen. The succinamide linkage of 1-amino TCDD and the activated ester method for covalent binding to protein will be investigated including ^{14}C labeling of the linkage for quantification purposes.

KEYWORD DESCRIPTORS: Radioimmunoassay (RIA), haptens and antigens of 1-amino-2,3,7,8-tetrachlorodibenzo-p-dioxin (1-NH₂ TCDD).

Project No.: Z01 ES 30010-01 EBCB

1. Environmental Biology and Chemistry Branch
2. Comparative Biology Section
3. Research Triangle Park, N.C.

PHS-NIH

Individual Project Report

July 1, 1974 through June 30, 1975

PROJECT TITLE: "Development of Immunoassay Techniques for Detection and Quantitation of Environmental Chemicals"

PERVIOUS SERIAL NUMBER: None

PRINCIPAL INVESTIGATOR: Robert E. Faith, D.V.M., M.S.

OTHER INVESTIGATORS: John A. Moore, D.V.M., J.D. McKinney, Ph.D., P.W. Albro, Ph.D., and K. Chae, Ph.D.

COOPERATING UNITS: None

MAN YEARS

Total:	0.3
Professional:	0.2
Other:	0.1

PROJECT DESCRIPTION

OBJECTIVE: To develop methodology for immunoassay of environmental chemicals.

METHODS EMPLOYED: Presently rabbits are being immunized with 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) - protein conjugates emulsified in complete Freund's adjuvant. Antibody production is being monitored by means of double diffusion in gel and capillary tube precipitation reactions. Other compounds will be introduced into the study. When sufficient antibodies are obtained they will be employed to develop methodology for immunoassay of environmental chemicals.

MAJOR FINDINGS: The study is in its early stages so there are no major findings.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Specific, highly sensitive assays for TCDD and other environmental chemicals would be very useful in studies of environmental contamination, body burden and distribution, etc., with these chemicals. This study will hopefully develop the methodology required for specific and sensitive assays for environmental chemicals.

PROPOSED COURSE: To be continued

KEYWORD DESCRIPTORS: Immunoassay; environmental chemicals.

Project No.: Z01 ES 30011-03 EBCB

1. Environmental Biology and Chemistry Branch
2. Chemistry Section
3. Research Triangle Park, N.C.

PHS-NIH

Individual Project Report

July 1, 1974 through June 30, 1975

PROJECT TITLE: "Synthesis and Chemistry of Chlorinated Aromatic and Oxygenated Chlorinated Aromatic Compounds and Their Environmental Transformation Products"

PREVIOUS SERIAL NUMBER: NIEHS-EBC-009-2 and part of terminated NIEHS-EBC-008

PRINCIPAL INVESTIGATOR: Louis A. Levy, Ph.D.

OTHER INVESTIGATORS: M. Walker, K. Chae, Ph.D. and J. D. McKinney, Ph.D.

COOPERATING UNITS: Comparative Biology Section, EBCB, NIEHS

MAN YEARS

Total: 0.9

Professional: 0.7

Other: 0.2

PROJECT DESCRIPTION

OBJECTIVES: To develop rational synthetic routes to chlorinated biphenyls, chlorinated dibenzofurans, prefurans and their metabolites, especially the assumed reactive intermediate arene oxides (oxepins) and penultimate hydroxylated derivatives. To investigate the chemical and physical properties of these compounds and their interaction-reaction products, which will aid in identifying them in the environment and assessment (rate and extent) of reactivity with biological entities.

METHODS EMPLOYED: Synthetic techniques, organometallic reagents and catalysis, high pressure reactions, photochemical processes, functional group transformations. Mass spectroscopy, other spectroscopic methods (IR, NMR), chromatography (column, GLC, liquid).

MAJOR FINDINGS: The conversion of 2,3,6-trichlorobenzoic acid to 2,3,6,2',3',6'-hexachlorobiphenyl, a constituent of commercial Aroclors, has been affected. This compound due to the position of the chlorine substituents is capable of being resolved into optical isomers. Some preliminary exploration into the possibility of selective destruction of one of the enantiomers of the racemic mixture by bacteriological action has been carried out. So far the results have been ambiguous.

The use of the Diels-Alder cycloaddition reaction has been successfully utilized

to synthesize appropriately functionalized intermediates which have been further transformed to derivatives of biphenyl oxides and unsymmetrically substituted chlorinated biphenyls.

Some preliminary work has been done on developing methodology for the synthesis of chlorinated dibenzofurans. One approach involves the introduction of the oxygen bridge as the ultimate step in the formation of the dibenzofuran nucleus. Another approach involves, as the last step, the joining together of the two phenyl nuclei through a carbon-carbon bond.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The commercial PCB's are known to be complex mixtures and are recognized to be of major environmental importance due to their widespread ecological distribution and their concentration in the food chain. Furthermore, chlorinated dibenzofuran impurities found along with PCB and/or produced from PCB by natural processes are known to be toxic. The availability of the unique isomers of these classes of compounds and their probable metabolites would permit more definitive biological and toxicological studies.

PROPOSED COURSE: To continue the synthesis of individual isomers of PCB's and dibenzofurans as well as their potential or actual metabolites such as arene oxides and hydroxylated derivatives. Especial attention is to be focused on the continuing use of the cycloaddition reaction and associated sequence mentioned above.

KEYWORD DESCRIPTORS: Chlorinated biphenyls; biphenyl stereoisomerism; Diels-Alder reaction; biphenyl oxide intermediates; dibenzofuran.

PUBLICATIONS

Levy, L. A.: A Convenient Preparation of Homogeneous 2,3,6-Trichlorobenzoic Acid and Its Conversion to 2,2',3,3',6,6'-Hexachlorobiphenyl. Organic Preparations and Procedures Int. 6: 275, 1974.

Project No.: Z01 ES 30012-02 EBCB

1. Environmental Biology and Chemistry Branch
2. Chemistry Section
3. Research Triangle Park, N.C.

PHS-NIH

Individual Project Report

July 1, 1974 through June 30, 1975

PROJECT TITLE: "Comparative Chemistry and Biological Properties of Selected Hexachlorobiphenyl Isomers"

PREVIOUS SERIAL NUMBER: NIEHS-EBC-010

PRINCIPAL INVESTIGATOR: James D. McKinney, Ph.D.

OTHER INVESTIGATORS: K. Chae, Ph.D., B. Gupta, B.V.Sc. & A.H., Ph.D., L. Levy, Ph.D., J. A. Moore, D.V.M., M. Walker and J. Goldstein, Ph.D.

COOPERATING UNITS: Comparative Biology Section, EBCB, NIEHS, and Pesticides and Toxic Substances Effects Lab, NERC, EPA, Research Triangle Park, NC

MAN YEARS

Total:	0.8
Professional:	0.7
Other:	0.1

PROJECT DESCRIPTION

OBJECTIVES: To begin to clarify and define the biological effects of commercial PCB-mixtures by systematically examining the chemistry and biological effects of individual selected PCBs from synthesis. To correlate the physical-chemical properties of selected hexachlorobiphenyls (HCBs) with toxicity.

METHODS EMPLOYED: The chemistry of the HCB isomers was defined using a variety of synthetic, chromatographic, and spectroscopic techniques. Such biological responses as mortality, body weight change, food consumption, body organ weights, incidence of edema and porphyria, liver enzyme induction, etc. were measured simultaneously.

MAJOR FINDINGS: The biological activity of five synthetic hexachlorobiphenyls [symmetrical isomers, 245, 236, 246, 234 and 345 (contained about 30% hepta)] and 2,3,7,8-tetrachlorodibenzofuran (TCDF) was investigated in the chick at a dietary level of 400 ppm (except 245 @ 100 or 400 ppm; TCDF @ 1 or 5 $\mu\text{g/kg}$) over a 21 day period. The 345 isomer was generally the most active (all chicks died) and the 236 the least. The HCB retention indices as measured by gas chromatography generally correlated with lipophilicity and overall toxicity. Physical-chemical data supported the importance of 3 (and 5) and 4 substitution

of chlorine for high polarity and lipophilicity.

More recently the pure 345 isomer was observed to be highly active (all chicks died) at 100 ppm in the diet with no gross toxicity apparent from a synthetic metabolite possibility (2-OH, 345) at 10 and 30 ppm. Toxicity data for this isomer suggests the possibility of metabolism to a chlorinated dibenzofuran.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The widespread use and occurrence of the polychlorinated biphenyls has necessitated investigations of their possible human health effects. In order to facilitate such studies, systematic examination of purified isomers is necessary to obtain clarity and definition of the biological effects.

PROPOSED COURSE: To continue studies with the chick to determine the minimum dietary dose levels of 345 HCB producing toxic symptoms. To delineate the metabolism of the 345 isomers with ^{14}C labeling. To extend studies to mammalian systems.

KEYWORD DESCRIPTORS: Hexachlorinated biphenyls; physical-chemical properties; toxicity correlations.

PUBLICATIONS

McKinney, J.D., Chae, K., Moore, J.A. and Gupta, B.: Toxicology of hexachlorobiphenyl isomers and 2,3,7,8-tetrachlorodibenzofuran in chicks. I. Relationship of chemical parameters. In preparation, 1975.

Goldstein, J.A., McKinney, J.D., Lucier, G.W., Hickman, P., Bergman, H. and Moore J.A.: Toxicology of hexachlorobiphenyl isomers and 2,3,7,8-tetrachlorodibenzofuran in chicks. II. Effects on drug metabolism and porphyrin accumulation. In preparation, 1975.

Project No.: Z01 ES 30013-01 EBCB

1. Environmental Biology and Chemistry Branch
2. Chemistry Section
3. Research Triangle Park, N.C.

PHS-NIH

Individual Project Report

July 1, 1974 through June 30, 1975

PROJECT TITLE: "Identification of Metabolites of Symmetric Hexachlorobiphenyls in the Chicken"

PREVIOUS SERIAL NUMBER: None

PRINCIPAL INVESTIGATOR: J. Ronald Hass, Ph.D.

OTHER INVESTIGATORS: J. D. McKinney, Ph.D., and K. C. Chae, Ph.D.

COOPERATING UNITS: None

MAN YEARS

Total: 0.1

Professional: 0.1

Other: 0.0

PROJECT DESCRIPTION

OBJECTIVES: To identify metabolites of selected hexachlorobiphenyls (HCB's) isolated from chicken excreta.

METHODS EMPLOYED: Extracts of excreta from chickens which had been fed 2,4,6,2',4',6'-HCB, 2,3,6,2',3',6'-HCB, 2,4,5,2',4',5'-HCB, 2,3,4,2',3',4'-HCB, or 3,4,5,3',4',5'-HCB were provided by Dr. Chae. These extracts were analyzed by means of gas chromatography (GC) and combined gas chromatography-mass spectrometry (GC-MS).

MAJOR FINDINGS: These preliminary findings indicated the need for further sample clean-up. Also, the presence of a pentachlorodibenzofuran was indicated in the 2,4,5,2',4',5'-HCB metabolite sample. Sample clean-up has been completed and the analysis by means of GC/MS will continue pending availability of instrument time.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: This project is part of a larger study at the Institute to determine the toxicity and metabolic fate of individual chlorinated biphenyls.

PROPOSED COURSE: To be continued.

KEYWORD DESCRIPTORS: Mass spectrometry; GC-MS; metabolite identification; hexachlorobiphenyls; chickens.

Project No.: Z01 ES 30014-01 EBCB

1. Environmental Biology and Chemistry Branch
2. Chemistry Section
3. Research Triangle Park, N.C.

PHS-NIH

Individual Project Report

July 1, 1974 through June 30, 1975

PROJECT TITLE: "Identification of Metabolites of 4-Chlorobiphenyl in the Rat"

PREVIOUS SERIAL NUMBER: None

PRINCIPAL INVESTIGATOR: J. Ronald Hass, Ph.D.

OTHER INVESTIGATORS: S. Matthews, Ph.D., and L. T. Jao, Ph.D.

COOPERATING UNITS: Pharmacology Branch, NIEHS, EPA

MAN YEARS

Total:	0.1
Professional:	0.1
Other:	0.0

PROJECT DESCRIPTION

OBJECTIVES: To identify the metabolites of 4'-chlorobiphenyl in the rat.

METHODS EMPLOYED: Samples of rat fecal and urine extract were analyzed by gas chromatography (GC) and combined gas chromatography-mass spectrometry (GC-MS) using electron impact (ei) and chemical ionization (ci). When practical, samples were also analyzed by proton and ^{13}C NMR.

MAJOR FINDINGS: A preliminary analysis of the metabolite samples by GC indicated the presence of four unique metabolites. Subsequent analysis by GC-MS showed these to be two isomeric monohydroxy monochlorobiphenyls and two isomeric dihydroxy monochlorobiphenyls. One of the monohydroxy monochlorobiphenyls has been identified as 4' hydroxy-4-chlorobiphenyl. Identification of the remaining metabolites is in progress.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: This project is part of a larger study at the Institute to determine the toxicity and metabolic fates of individual chlorinated biphenyls.

PROPOSED COURSE: To be continued.

KEYWORD DESCRIPTORS: Mass spectrometry; GC/MS; metabolite identification; 4' chlorobiphenyl; rats.

Project No.: Z01 ES 30015-01 EBCB
1. Environmental Biology and Chemistry Branch
2. Chemistry Section
3. Research Triangle Park, N.C.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

PROJECT TITLE: "Studies in Chemical Ionization Mass Spectrometry"

PREVIOUS SERIAL NUMBER: None

PRINCIPAL INVESTIGATOR: J. Ronald Hass, Ph.D.

OTHER INVESTIGATORS: Mr. W. B. Nixon

COOPERATING UNITS: The University of North Carolina at Chapel Hill, Department
of Chemistry, UNC-CH

MAN YEARS

Total:	0.1
Professional:	0.1
Other:	0.0

PROJECT DESCRIPTION

OBJECTIVES: The aim of this project is to develop qualitative analytical methods applicable to biomedical samples based on the use of selective reagent gases in chemical ionization mass spectrometry (cims). It is anticipated that such methods will in turn improve our capabilities in the area of sample identification when insufficient material is available for other methods to be used.

METHODS EMPLOYED: The high pressure mass spectrometer in the NIEHS spectrometry center is being used in this work. Biacetyl was chosen as the first compound for study in view of the literature available describing its gaseous ion chemistry. Studies of ion abundance as a function of pressure and the ion cyclotron resonance mass spectrometer (icv) at UNC-CH are being used to deduce the mechanism of reactions observed.

MAJOR FINDINGS: This work is in a very early stage of development. Preliminary experiments indicated that diacetyl is suitable for further evaluation as a source of reagent ions for CiMS in that the background ion current is composed predominately of three ions [CH_3CO^+ ; $(\text{CH}_3\text{CO})_2\text{H}^+$; and $(\text{CH}_3\text{CO})_3^+$]. Furthermore, the reactivity of biacetyl under cims conditions appears to be similar to that observed earlier in the icv work. These findings are being substantiated at present.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The application of mass spectrometry to problems in biomedical analysis in recent

years has been particularly fruitful. Chemical ionization techniques have provided information complimentary to data acquired using electron impact ionization methods. This project should allow a means to gather further information concerning the nature of an unknown sample.

PROPOSED COURSE: To be continued.

KEYWORD DESCRIPTORS: Chemical ionization mass spectrometry; biacetyl.

Project No.: Z01 ES 30016-03 EBCB

1. Environmental Biology and Chemistry Branch
2. Chemistry Section
3. Research Triangle Park, N.C.

PHS-NIH

Individual Project Report

July 1, 1974 through June 30, 1975

PROJECT TITLE: "Applications of Nuclear Magnetic Resonance to (1) Molecular and Electronic Structure Determinations and (2) The Study of Model Membrane Systems and Other Biopolymer Association and Equilibria"

PREVIOUS SERIAL NUMBER: NIEHS-EBC-021, 013-2, 032-2 and 033-2

PRINCIPAL INVESTIGATOR: James D. McKinney, Ph.D.

OTHER INVESTIGATORS: V. Mudgal, Ph.D., J. Memory, Ph.D., W. Fleming and N. Wilson, Ph.D.

COOPERATING UNITS: Department of Physics, N.C. State University and Pesticides and Toxic Substances Effects Lab, NERC, EPA, Research Triangle Park, NC

MAN YEARS

Total:	1.0
Professional:	0.9
Other:	0.1

PROJECT DESCRIPTION

OBJECTIVES: To routinely examine the molecular and electronic structure of environmental agents and their transformation products under study at the institute. To use NMR to determine the microenvironments of these compounds in complex biological and non-biological matrices.

METHODS EMPLOYED: Variable temperature NMR and supportive computer methodology.

MAJOR FINDINGS: The NMR facility responded to 74 requests for service which included over 100 samples covering a variety of classes of chemical compounds. A variety of techniques were employed to analyze these samples including ¹H and ¹³C analysis, spin decoupling experiments, addition of shift reagents to aid in establishing the position and stereochemistry of functional groups, etc. Only a few of these compounds were authentic metabolites, and the remainder were commercially available or synthetic chemical standards. The diverse nature of compounds and the types of information sought during their analysis continue to broaden our capability in this area.

Preliminary studies with long chain aliphatic hydrocarbon alcohols have indicated

that NMR and shift reagents may be useful in determining the environment of hydroxyl groups and, hence, points of attack by hydroxylating enzyme systems.

^{13}C -NMR has confirmed the deshielding effects that arise when lipophilic compounds such as bis(p-chlorophenyl) acetic acid (DDA) are solubilized in lecithin-benzene-water reversed micelles. This supports the proposal that such molecules aggregate in this environment via electrostatic interactions.

Preliminary studies with ^{13}C -enriched benzene has indicated that NMR may be useful in at least, qualitatively determining metabolism via analysis of body fluids and tissues or their crude extracts and homogenates. For example, urine from benzene treated animals can be examined directly for the presence of phenol and catechol.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The NMR facility will continue to provide a basic analytical service for many research programs. Research studies with NMR are aimed at correlating the physical-chemical properties of environmental agents with such biologically important factors as lipophilicity and persistence, metabolic propensity, conjugatability/excreatability of transformation products and mechanisms of carcinogenicity and mutagenicity.

PROPOSED COURSE: ^{13}C -enriched compounds such as in situ generated benzene oxide and the stable 4,5-epoxide of benzo[a]pyrene will be used in studies to investigate their role in carcinogenicity. ^{13}C -enriched 2,3,7,8-tetrachlorodibenzo-p-dioxin will be used in studies to qualitatively determine its metabolism in rats. Other studies will concentrate on mechanisms of lipophilicity.

KEYWORD DESCRIPTORS: Nuclear magnetic resonance; FT-NMR; regio- and stereospecificity in metabolism; mechanisms of lipophilicity and carcinogenicity; ^{13}C -enriched compounds.

Project No.: Z01 ES 30017-02 EBCB

1. Environmental Biology and Chemistry Branch
2. Chemistry Section
3. Research Triangle Park, N.C.

PHS-NIH

Individual Project Report

July 1, 1974 through June 30, 1975

PROJECT TITLE: "Biomimetic Models for the Study of Dechlorination Processes of Pesticides"

PREVIOUS SERIAL NUMBER: Part of terminated NIEHS-EBC-021

PRINCIPAL INVESTIGATOR: Louis A. Levy, Ph.D.

OTHER INVESTIGATORS: J. D. McKinney, Ph.D. and M. Walker

COOPERATING UNITS: As needed

MAN YEARS

Total:	0.8
Professional:	0.6
Other:	0.2

PROJECT DESCRIPTION

OBJECTIVES: To develop dechlorination reactions whose mechanism is understood and which involve defined intermediates. Analysis of the products of the dechlorination of target pesticide molecules and comparison with the results of environmental and/or biological dechlorination will aid in clarifying the mechanism of environmental degradation.

METHODS EMPLOYED: Photochemical processes, metal hydride reduction, gamma radiation. Preparation of standards by unambiguous synthetic methods. Mass spectroscopy, NMR spectroscopy. Chromatography (GLC, thin layer, column).

MAJOR FINDINGS: A number of chlorinated pesticides and derivatives obtained from them by environmental action have been dechlorinated using chemical (metal hydride) and physical (ultraviolet irradiation, gamma ray irradiation) systems known to involve free radical intermediates. The stereochemistry and regio-specificity of reductive dechlorination as a function of the reducing system as well as the hybridization of the carbon atom being reduced have been determined.

Some of these findings are at variance with published reports involving similar systems, however, we feel that our more complete data will clear up this apparent discrepancy. In some of these instances we have observed remarkable stereochemical control of dechlorination of the bridged polycyclic pesticides. A convenient method for obtaining pure, substantial quantities of heretofore rare isomers

of dechloro-pesticides has been developed. At least one set of chemical dechlorinating conditions has led to partial destruction of the basic skeletal system of photodieldrin affording a compound containing ethylenic chloride which resembles the vinyl chloride function.

A preliminary study of the oxidative dechlorination propensity of syn-dechlorodieldrin has been carried out in vivo in view of the possible formation of a chlorinated benzene. The results so far have been ambiguous.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The chlorinated pesticides are recognized to be major environmental concern due to their widespread distribution, their persistence and their concentration in the food chain. They are slowly converted to lesser chlorinated species, about which little is known. Some of these compounds represent radically new structural types. A knowledge of the processes by which these transformations take place would make possible more comprehensive biological and toxicological strides.

PROPOSED COURSE: To continue the study of the correlation of laboratory models of dechlorination with environmental processes. To develop reliable synthetic methods to produce rare and important isomers of pesticides of importance to other ongoing investigations.

KEYWORD DESCRIPTORS: Dechlorination reactions; gamma irradiation; metal hydride reduction; stereochemistry; nuclear magnetic resonance; chlorinated pesticides; photochemical transformation.

Project No.: Z01 ES 30018-02 EBCB

1. Environmental Biology and Chemistry Branch
2. Chemistry Section
3. Research Triangle Park, N.C.

PHS-NIH

Individual Project Report

July 1, 1974 through June 30, 1975

PROJECT TITLE: "Ester Hydrolases at the Environmental Interface"

PREVIOUS SERIAL NUMBER: NIEHS-EBC-024

PRINCIPAL INVESTIGATOR: Phillip W. Albro, Ph.D.

OTHER INVESTIGATORS: A. Latimer, B. Moore, B. J. Corbett

COOPERATING UNITS: None

MAN YEARS

Total:	0.70
Professional:	0.30
Other:	0.40

PROJECT DESCRIPTION

OBJECTIVES: To evaluate the capabilities of various tissue lipases and esterases as early mediators in the interaction of animals with xenobiotics containing ester linkages.

METHODS EMPLOYED: Most studies will involve the rat, but significant findings will be checked in mice, hamsters, guinea pigs and rabbits. Lung, gastrointestinal, and skin enzymes will be studied, using techniques generally described previously (Biochim. Biophys. Acta, 360: 380, 1973). Test substances will initially include: (1) esters of 2,4-D and 2,4,5-T, (2) phthalate plasticizers, (3) detergents such as Tween 20, Tween 80, (4) aromatic rubefacients such as methyl paraben, methyl salicylate, (5) volatile solvents such as ethyl acetate, butyl acetate, (6) carbamates.

MAJOR FINDINGS: (1) Gross evaluations of the relative abilities of rat lung, skin and pancreas homogenates to demonstrate aliesterase, aromesterase, emulsion lipase, micelle lipase, cholesteryl esterase, and lipoprotein lipase activities have been completed. Each tissue shows a distinctive pattern of such activities. (2) We have succeeded in developing a procedure for the purification of micelle lipase from rat pancreas, and its characterization is in progress. (3) Carboxyl ester hydrolysis by micelle lipase appears to involve a combination of B al 2 and A al 2 mechanisms. This is being studied using substituted phthalates as substrates in the presence of ^{18}O -enriched water. (4) The apparent K_m of micelle lipase is apparently independent of the nature of the ester substrate, and is dependent only on the type of bile salt used to form

micelles. V_{max} , however, is dependent upon the substrate.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Relatively little is known about non-specific lipase activity, since the enzyme(s) has(have) never been purified. Although a great deal is known about lung esterases, very little is known about lung lipases (hydrolases for insoluble esters). Virtually nothing is known about ester hydrolases in skin. These enzymes may either protect against some hazardous compounds by converting them to less easily absorbed, more polar materials, or increase the hazard of other compounds when the acid (or alcohol) is more toxic than the ester. A second anticipated result of this project should be a better understanding of how enzymes in general are able to bind to and react with water-insoluble compounds.

PROPOSED COURSE: Once the characterization of micelle lipase is completed, an attempt will be made to insolubilize it as a general reagent for esterolysis, since it is able to hydrolyze many otherwise refractory esters to completion. Attention will next be directed to either lung or skin enzymes, to determine their capacities for hydrolysis of xenobiotic esters in situ.

KEYWORD DESCRIPTORS: Non-specific lipase; micelle lipase; ester hydrolases; environmental interface; esters; lung, gastrointestinal, and skin esterases.

PUBLICATIONS

Albro, P.W. and Latimer, A.D.: Non-specific lipase: An enzyme highly specific for micelles. Biochemistry 13: 1431-1438, 1974.

Project No.: Z01 ES 30019-03 EBCB

1. Environmental Biology and Chemistry Branch
2. Chemistry Section
3. Research Triangle Park, N.C.

PHS-NIH

Individual Project Report

July 1, 1974 through June 30, 1975

PROJECT TITLE: "Hydrocarbons as Model Compounds for Metabolic Studies"

PREVIOUS SERIAL NUMBER: NIEHS-EBC-022-2

PRINCIPAL INVESTIGATOR: Phillip W. Albro, Ph.D.

OTHER INVESTIGATORS: R. Thomas, B. Moore

COOPERATING UNITS: None

MAN YEARS

Total:	0.15
Professional:	0.10
Other:	0.05

PROJECT DESCRIPTION

OBJECTIVES: To determine to what extent hydrocarbons may be used as model compounds in predicting metabolic modification of aliphatic chains in other classes of compounds.

METHODS EMPLOYED: The basic test organism is the rat, to which hydrocarbons are administered orally in corn oil, mixed with rat chow, injected I.P., I.V. or I.M. In some cases radioactive tracers (1-¹⁴C-hexadecane, 1-¹⁴C-octadecane, and U-¹⁴C-phytane and 2,3-³H-phytane synthesized here) are used in metabolic studies. Standard chromatographic, immunologic, radioassay and other biochemical techniques are used, except that balance studies of absorption from the gut are made as described in Biochim. Biophys. Acta 219: 437-446, 1970.

MAJOR FINDINGS: Rats metabolize phytane (tetramethyl-hexadecane) by two routes, one of which leads to the accumulation of 2,6,10,14-tetramethylhexadecan-2-ol in liver. The major pathway leads to the formation of C₂-C₆ carboxylic acids which are excreted in the urine. We were unable to detect lipoidal intermediates in spite of various "trapping" procedures, and concluded that the initial oxidation step was rate-limiting. This study has been published.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Biological mechanisms for the modification and degradation of aromatic, alicyclic and aliphatic moieties are far from adequately understood. An understanding of in vivo capabilities in these areas will simplify metabolic studies of many classes of compounds, and hopefully permit reliable predictions as to what types of

metabolites might be expected upon ingestion of various new drugs, pesticides, food additives, etc.

PROPOSED COURSE: In vitro studies are planned in an attempt to identify early metabolites in the degradation of isoprenoid hydrocarbons, since we feel that the early metabolites may be related to the adverse effects of these hydrocarbons on the immunologic competence in animals. The mechanism of attack at points of chain-branching, the new degradative pathway mentioned above, will be evaluated as to its generality and applicability to classes of compounds of greater environmental interest than hydrocarbons.

KEYWORD DESCRIPTORS: Hydrocarbons; model compounds, metabolism of alkyl chains; isoprenoid hydrocarbons; immunologic competence; food additives; phytane.

PUBLICATIONS

Albro, P.W. and Thomas, R.O.: Metabolism of phytane in rats. Biochim. Biophys. Acta 372: 1-14, 1974.

Albro, P.W. and Thomas, R.O.: Intestinal absorption of hexachlorobenzene and hexachlorocyclohexane isomers in rats. Bull. Env. Contam. Toxicol. 12: 289-294, 1974.

Project No.: Z01 ES 30020-04 EBCB

1. Environmental Biology and Chemistry Branch
2. Chemistry Section
3. Research Triangle Park, N.C.

PHS-NIH

Individual Project Report

July 1, 1974 through June 30, 1975

PROJECT TITLE: "Transport and Metabolism of Phthalate Esters"

PREVIOUS SERIAL NUMBER: NIEHS-EBC-023-3

PRINCIPAL INVESTIGATOR: P. W. Albro, Ph.D.

OTHER INVESTIGATORS: B. J. Corbett, R. Hass

COOPERATING UNITS: None

MAN YEARS

Total:	0.25
Professional:	0.20
Other:	0.05

PROJECT DESCRIPTION

OBJECTIVES: To prepare purified standard labeled and unlabeled phthalate esters, e.g., DHP [di(2-ethylhexyl)phthalate] and DOP-(dioctyl phthalates); and to elaborate aspects of their transport, absorption, excretion and tissue residues following various routes of administration, e.g., oral, inhalation and i.p., in the rat.

METHODS EMPLOYED: Chromatography (TLC, column, GLC), spectrophotometry (I.R., U.V., visible), mass spectrometry and isotopic methods, standard enzymology techniques.

MAJOR FINDINGS: (1) 2-Ethyl hexanol is efficiently absorbed, rapidly metabolized, and eliminated as expired CO₂ and various urinary metabolites by rats. Elimination is complete by 30 hours after an oral dose of up to 300 mg/Kg. The metabolites are derived by oxidation (alcohol dehydrogenase and aldehyde oxidase) to 2-ethylhexanoic acid followed by ω - and partial β -oxidation, as well as oxidative decarboxylation. The toxicity of ethylhexanol is suggested to be due to a persistent alteration of the NAD/NADH ratio in tissues, as has been suggested previously for ethanol toxicity. Results of this study have been submitted for publication. (2) Various substituted phthalates (dimethyl phthalate, di-n-butyl and di-n-octyl phthalates, di-2-ethyl hexyl phthalate, diethylhexyl 4-nitrophthalate and diethylhexyl tetrachlorophthalate) have been compared as substrates for the nonspecific lipase of rat pancreas. The tetrachlorophthalate can not be hydrolyzed by this enzyme.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Our previous studies had indicated that no more than traces of phthalate diester plasticizer would be absorbed intact from the intestinal tract of several species of animals. Non-specific ester hydrolases of ubiquitous distribution should eliminate the majority of those traces, for the water-insoluble diesters. The phthalate-containing hydrolytic products and their oxidative metabolites are rapidly excreted. Accordingly, the only significant hazard from orally ingested phthalates of this type, if indeed there is a hazard, would probably be associated with the alcohol hydrolysis product. This hazard may be predictively evaluated, for chronic toxicity at least, by combining data on LD₅₀ for the alcohol moieties with data on rates of hydrolysis of the diesters. In addition, present work suggests that an evaluation of the toxicities of ortho disubstituted phthalates (as diesters) might be desirable, since they may be resistant to hydrolysis by intestinal enzymes.

PROPOSED COURSE: The ability of the lung lipases to hydrolyze phthalate plasticizers presented as vapors or aerosols remains to be studied.

KEYWORD DESCRIPTORS: Transport; intermediary metabolism; phthalate esters; 2-ethylhexanol; absorption; substituted phthalates.

PUBLICATIONS

Albro, P.W. and Moore, B.: Identification of the metabolites of simple phthalate diesters in rat urine. J. Chromatogr. 94: 209-218, 1974.

Project No.: Z01 ES 30021-02 EBCB

1. Environmental Biology and Chemistry Branch
2. Comparative Biology Section
3. Research Triangle Park, N.C.

PHS-NIH

Individual Project Report

July 1, 1974 through June 30, 1975

PROJECT TITLE: "Effects of Environmental Compounds on Intracerebral Tumor Induction by Avian Sarcoma Virus"

PREVIOUS SERIAL NUMBER: NIEHS-EBC-016

PRINCIPAL INVESTIGATOR: Dana D. Copeland, M.D.

OTHER INVESTIGATORS: None

COOPERATING UNITS: Darell D. Bigner, M.D., Ph.D., Department of Pathology, Duke University

MAN YEARS

Total:	0.7
Professional:	0.6
Other:	0.1

PROJECT DESCRIPTION

OBJECTIVES: To define a suitable model for tumor induction in laboratory animals by a transmissible biological agent and to assess any effect of selected environmental compounds on the oncogenic activity of the agent.

METHODS EMPLOYED: The Bratislava - 77 strain of avian sarcoma virus (B-77-ASV) is grown in tissue culture of chick embryo fibroblasts. Virus is isolated and then concentrated by filtration and centrifugation. Inocula of virus on citrate buffer are delivered by microliter syringe intracerebrally into fetal, neonatal, and adult Fischer 344 rats. Environmental agents will be administered before and/or after administration of virus. Following treatments tumor incidence and latency and tumor related mortality is recorded. The morphology of induced tumors is studied by conventional light microscopy, transmission electron microscopy, and scanning electron microscopy.

MAJOR FINDINGS: B-77-ASV induces cerebral tumors in adult as well as perinatal rats. The percentage of tumor induction and the mean number of tumors per animal decreases as the age of the animal at inoculation increases. Tumor related mortality is most precipitous in rats inoculated at younger ages and is comparable among male and female animals of the same age. The length of the latent period increases with increasing age of the animal at inoculation and decreasing titer of the viral inoculum. At very low titers the latent period may exceed the normal lifespan of the animal.

A large majority of tumors induced in all age groups are glial; the remainder are mesenchymal and mixed glial and mesenchymal. Inoculation in utero and at one day of age induces mixed gliomas with populations of oligodendroglia, astrocytes, and spongioblasts. Tumors in groups inoculated as adults include gemistocytic, pilocytic, fibrillary and anaplastic astrocytomas, and only rare mixed tumors. Tumor morphology is more uniform in rats inoculated as adults. No neuronal tumors occur even in animals inoculated in utero.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Environmental agents which may not in themselves be oncogenic may potentiate oncogenic viruses. An evaluation of suppression of immune surveillance mechanisms which may regulate the oncogenic activity of a virus is an important part of the assessment of any potentially hazardous agent. The experimental model described above provides an efficacious technique for the evaluation of such effects.

PROPOSED COURSE: Experiments are underway to determine any differences in the tumor incidence and post-inoculation survival between males, females, and castrated males inoculated as adults and as neonates. A group of rats of 18 months age has been inoculated so that the relationship between tumor incidence and latency may be extended into the final months of the normal Fischer rat life span. Experiments to determine the effect of environmental agents on the induction of CNS tumors by B-77-ASV are being designed.

KEYWORD DESCRIPTORS: Avian sarcoma virus; Rous sarcoma virus; brain tumor model; neoplasms; gliomas; chemotherapy; immunotherapy; electron microscopy.

PUBLICATIONS

Copeland, D.D., Vogel, F.S., and Bigner, D.D.: The induction of intracranial neoplasms by the inoculation of avian sarcoma virus in perinatal and adult rats. J. Neuropath. & Exp. Neurol. (In press), 1975.

Project No.: Z01 ES 30022-01 EBCB

1. Environmental Biology and Chemistry Branch
2. Comparative Biology Section
3. Research Triangle Park, N.C.

PHS-NIH

Individual Project Report

July 1, 1974 through June 30, 1975

PROJECT TITLE: "Pathogenesis of Hemorrhagic Cyst Disease in Fischer Rats"

PREVIOUS SERIAL NUMBER: None

PRINCIPAL INVESTIGATORS: Donald B. Feldman, D.V.M., Robert E. Faith, D.V.M., Ph.D. and Dana D. Copeland, M.D.

OTHER INVESTIGATORS: Ernest E. McConnell, D.V.M.

COOPERATING UNITS: Darell D. Bigner, M.D., Ph.D., Department of Pathology, Duke University

MAN YEARS

Total:	0.6
Professional:	0.3
Other:	0.3

PROJECT DESCRIPTION

OBJECTIVES: To define the pathogenesis of hemorrhagic cyst disease and evaluate the usefulness of the phenomena as an animal disease model.

METHODS EMPLOYED: Neonatal Fischer rats are inoculated intracranially with the Bratislava-77 strain of avian sarcoma virus (B-77-ASV). The evolution of hemorrhagic cysts is studied by sequential autopsy and fine dissection of cysts. Morphologic features are studied by light microscopy, transmission electron microscopy, and scanning electron microscopy. Cyst fluid is studied by conventional clinical pathologic methods. Cell mediated immunity is being assessed in vitro by the response of thymic or splenic cell cultures to the mitogens Concanavalin A or phytohemagglutinin and in vivo by delayed hypersensitivity responses to the contact sensitizing agent 4-ethoxy-methylene-2-phenyl-2-oxazolone. Humoral immunity is being assessed by passive hemagglutination to measure antibody response to both T-independent and T-dependent antigens.

MAJOR FINDINGS: In rats inoculated on the first post-natal day cysts begin appearing as early as 21 days and may continue to develop for several months. Cysts are most common in the distribution of the superficial lymph nodes of the neck and the deep lymph nodes of the retroperitoneum. Morphologically the cysts are lined by endothelium and appear to be multivesicular and may contain granulation tissue. Hemorrhagic lymph nodes are common in the region around hemorrhagic cysts. Animals with cysts show suppression in the response of both

thymic and splenic lymphocytes to stimulation with Con A and a suppression in the response of thymic lymphocytes to stimulation with phytohemagglutinin. In both cases the response of lymphocytes from animals with cyst and virus induced tumors is somewhat more suppressed than the response of animals with virus induced tumors but without cysts.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Hemorrhagic cyst disease is a phenomena without known parallel in comparative medicine. Because of the implications that immune parameters are involved in the pathogenesis of the disease the disorder may prove to be a useful and unique model for the investigation of effects of environmental agents on immunologically compromised animals.

PROPOSED COURSE: Further investigations planned or now being conducted include: microdissection of cysts and contiguous vasculature, study of the cyst wall by transmission electron microscopy and scanning electron microscopy, and comparison of immune competency in those animals developing cysts to animals that do not develop cysts and to normal uninoculated animals.

KEYWORD DESCRIPTORS: Hemorrhagic cyst disease; avian sarcoma virus; Rous sarcoma virus; lymph nodes; cell mediated immunity; humoral immunity; thymus; lymphangioma; electron microscopy.

Project No.: Z01 ES 30023-01 EBCB

1. Environmental Biology and Chemistry Branch
2. Comparative Biology Section
3. Research Triangle Park, N.C.

PHS-NIH

Individual Project Report

July 1, 1974 through June 30, 1975

PROJECT TITLE: "Morphological Parameters of Glial Cell Lines Grown in Matrix Culture"

PREVIOUS SERIAL NUMBER: None

PRINCIPAL INVESTIGATOR: Dana D. Copeland, M.D.

OTHER INVESTIGATORS: None

COOPERATING UNITS: Darell D. Bigner, M.D., Ph.D., Department of Pathology,
Duke University

MAN YEARS

Total:	0.5
Professional:	0.3
Other:	0.2

PROJECT DESCRIPTION

OBJECTIVES: To characterize morphological markers which distinguish glial from non-glial and transformed from non-transformed cells in matrix cultures.

METHODS EMPLOYED: Cell lines derived from: avian sarcoma induced rat brain tumors, chemically induced rat brain tumors, rat fibroblasts, ASV-induced rat sarcomas and human brain tumors will be studied in three dimensional cultures grown on gelatin foam matrix. Cell morphology will be studied by conventional light microscopy, transmission electron microscopy and scanning electron microscopy. Glial markers will be verified by indirect membrane immunofluorescent assay for glial fibrillary acidic protein and S-100 protein.

MAJOR FINDINGS: Preliminary studies support the feasibility of the experimental design. Results with formalin fixation and paraffin embedding reveal that cells in matrix culture have similar morphology and staining properties to analogous cells *in vivo*. Ultrastructural studies by transmission electron microscopy suggest that the frequency of 80-90 Å filaments and gap junctions are greater in glial cells grown in matrix culture, while studies by scanning electron microscopy suggest that the frequency and configuration of microvilli may be characteristic of ASV transformed cells in matrix culture.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Tissue culture techniques have been applied in a wide range of investigations in

neurobiology. A recurrent problem in the application of such techniques has been the difficulty in identifying glial cells with characteristic glial morphology in dispersed cell cultures. The development of suitable techniques for the in vitro study of glial tissue should permit investigations in a number of areas such as the effects of environmental agents on kinetic cycles, metabolic pathways, and organogenesis in glial tissue.

PROPOSED COURSE: Work is in progress to characterize the morphological markers outlined above both by scanning and electron microscopy.

KEYWORD DESCRIPTORS: Tissue culture; matrix culture; glial; electron microscopy; scanning electron microscopy; gelfoam; glial filaments; nexus; microvilli.

Project No.: Z01 ES 30024-01 EBCB

1. Environmental Biology and Chemistry Branch
2. Comparative Biology Section
3. Research Triangle Park, N.C.

PHS-NIH

Individual Project Report

July 1, 1974 through June 30, 1975

PROJECT TITLE: "Primate Disease Investigation"

PREVIOUS SERIAL NUMBER: None

PRINCIPAL INVESTIGATOR: Ernest E. McConnell, D.V.M.

OTHER INVESTIGATORS: John A. Moore, D.V.M.

COOPERATING UNITS: J. Ronald Hass, Ph.D., Chemistry Section, EBCB, Fred Talley, Histology Section, EBCB

MAN YEARS

Total:	0.4
Professional:	0.3
Other:	0.1

PROJECT DESCRIPTION

OBJECTIVES: To investigate and define a disease syndrome affecting the Rhesus monkey breeding colony at Perrine, Florida since it resembles polychlorinated biphenyl (PCB) intoxication. If possible, to determine the etiology.

METHODS EMPLOYED: On site investigation of the problem. Epidemiological investigation of the disease syndrome with comparison to similar problems at other primate facilities. Necropsy and clinicopathologic determinations on affected animals. Radiographic and histopathologic work-ups on necropsied animals. Spectrophotometric analyses of animal tissues and suspect environmental compounds.

MAJOR FINDINGS: Monkeys in certain cages are more affected than in other areas. The disease syndrome is confined to epithelial tissues with lesions characteristically found in the skin, toenails, eyelids, oral cavity, facial bones, salivary glands, mammary gland, stomach and colon. The essential lesion is metaplasia and/or hyperplasia resembling vitamin A deficiency. Liver and serum Vit. A levels are lower than those in similar age Rhesus monkeys from a different geographic local. These findings suggest chronic toxicity by a compound such as a polycyclic chlorinated hydrocarbon whose effect is mediated through a deficiency of Vit. A. However, no significant amounts of PCB's were found in animal tissue.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Primate breeding facilities are becoming progressively more important as foreign suppliers of these animals diminish. The usefulness of the facility depends on determining and hopefully eliminating the cause of the problem. The lesions shown by affected animals suggest an environmental toxicologic factor, possibly a polycyclic chlorinated hydrocarbon. NIEHS has valuable experience and expertise with these compounds and therefore is well suited to investigate this aspect of the problem.

PROPOSED COURSE: To be continued.

KEYWORD DESCRIPTORS: Primate disease etiology; similarity to PCB intoxication; epidemiological investigation; environmental toxicologic factor.

Project No.: Z01 ES 30025-02 EBCB

1. Environmental Biology and Chemistry Branch
2. Comparative Biology Section
3. Research Triangle Park, N.C.

PHS-NIH

Individual Project Report

July 1, 1974 through June 30, 1975

PROJECT TITLE: "Carcinogenicity of Trisodium Nitritotriacetate (NTA) in the Rat"

PREVIOUS SERIAL NUMBER: NIEHS-EBC-019

PRINCIPAL INVESTIGATOR: Robert A. Goyer, M.D., University of North Carolina, Chapel Hill

OTHER INVESTIGATORS: Donald B. Feldman, D.V.M.

COOPERATING UNITS: Comparative Biology Section, EBCB and Clinical Laboratories, Memorial Hospital, University of North Carolina, Chapel Hill, N.C.

MAN YEARS

Total:	0.5
Professional:	0.5
Other:	0.0

PROJECT DESCRIPTION

OBJECTIVES: To determine the incidence of tumors in rats fed low levels of NTA in the drinking water throughout most of their life.

METHODS EMPLOYED: Male Sprague Dawley rats (50-70 g) were divided into 2 groups of approximately 200 animals each and were housed individually in suspended cages in a barrier sustained room. The control group received deionized drinking water containing 0.1% NTA (100 ppm). Those rats that died, were moribund, or continually lost weight during the experimental period were necropsied. Histopathologic examination was performed on major organs to determine the cause of death or morbidity. Rats with discernible tumors were also necropsied. Approximately 26 months after commencement of the experiment, all survivors were killed and necropsied as above. Blood samples were taken from approximately 180 control or experimental rats for selected clinical chemistry determination. Femurs and sternae from 40 rats were frozen, to be subsequently analyzed for NTA content.

MAJOR FINDINGS: Experimental rats had significantly greater renal lesions characterized by tubular hyperplasia, and resembling adenomas in a number of cases.

Chemical determinations on rat sera did not reveal differences between experimental and control groups.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: NTA usage in commercial detergents has been delayed in this country until definitive studies determine its safety, including assessment as to carcinogenic potential. The results of this study hopefully will contribute to the final decision.

PROPOSED COURSE: This project is completed. The data is now being analyzed for subsequent publication.

KEYWORD DESCRIPTORS: NTA; carcinogenesis; chelating agents.

Project No.: Z01. ES 30026-01 EBCB

1. Environmental Biology and Chemistry Branch
2. Comparative Biology Section
3. Research Triangle Park, N.C.

PHS-NIH

Individual Project Report

July 1, 1974 through June 30, 1975

PROJECT TITLE: "The Histopathologic Effects of Euthanasia on Laboratory Animals"

PERVIOUS SERIAL NUMBER: None

PRINCIPAL INVESTIGATOR: Donald B. Feldman, D.V.M.

OTHER INVESTIGATORS: Bholu N. Gupta, B.V.Sc.& A.H., Ph.D.

COOPERATING UNITS: None

MAN YEARS

Total: 0.4

Professional: 0.3

Other: 0.1

PROJECT DESCRIPTION

OBJECTIVES: To determine the histopathologic changes that result from various methods of euthanasia in mice, rats, guinea pigs and rabbits.

METHODS EMPLOYED: Groups of 4 animals of each species were killed according to commonly employed methods which included decapitation (mice, rats, guinea pigs), cervical dislocation (mice), CO₂, air embolism (rabbits) and various parenteral injections of sodium pentobarbital. Animals were necropsied immediately after death and tissues were processed for microscopic examination.

MAJOR FINDINGS: Most methods of euthanasia allowed good visualization of abdominal viscera, except concentrated sodium pentobarbital injected intraperitoneally. This drug was caustic to the serosal surface of underlying organs and caused necrosis of capsular and subserosal cells. Most methods of euthanasia caused variable pulmonary vascular congestion but usually the lung architecture was not distorted. Euthanasia with CO₂ or sodium pentobarbital injected intraperitoneally or intravenously affected the lungs minimally. Concentrated sodium pentobarbital injected into the right ventricle of the heart caused hemolysis of erythrocytes and edema of both pulmonary arterial walls and alveoli.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The method of euthanasia merits careful deliberation in the design of experiments.

If histopathologic interpretation must be made, it is important that euthanasia does not distort cellular architecture.

PROPOSED COURSE: This project has been completed and the data is being compiled for publication.

KEYWORD DESCRIPTORS: Euthanasia; histopathology; laboratory animal medicine.

Project No.: Z01 ES 30027-02 EBCB

1. Environmental Biology and Chemistry Branch
2. Comparative Biology Section
3. Research Triangle Park, N.C.

PHS-NIH

Individual Project Report

July 1, 1974 through June 30, 1975

PROJECT TITLE: "Evaluation of a Combined Pasteurella - Bordetella Autogenous Bacterin in Rabbits"

PREVIOUS SERIAL NUMBER: NIEHS-EBC-020

PRINCIPAL INVESTIGATOR: J. E. Thigpen, Ph.D.

OTHER INVESTIGATORS: Donald B. Feldman, D.V.M., and John A. Moore, D.V.M.

COOPERATING UNITS: Pel-Freez Rabbitry; Animal Husbandry Section, EBCB

MAN YEARS

Total:	0.5
Professional:	0.2
Other:	0.3

PROJECT DESCRIPTION

OBJECTIVES: To produce and evaluate a combined Pasteurella multocida - Bordetella bronchiseptica autogenous bacterin against respiratory disease ("snuffles") in rabbits.

METHODS EMPLOYED: Pasteurella multocida (Serotypes A, B, D, E) and Bordetella bronchiseptica isolates from infected New Zealand White rabbits were grown separately in trypticase soy broth. Total counts were determined on each serotype after which they were formalized and tested for sterility and toxicity. Approximately 200 cc of bacterin and control (placebo) respectively were submitted to the rabbitry to be administered in a blind study to female weanling rabbits 8 weeks old. The initial 2 cc vaccination was followed by 1 cc 2 weeks later. These animals were appropriately tattooed A or B depending upon the vial label (A=vaccine; B=placebo) and allowed to mature until 6 months of age. Upon return to NIEHS, they will be cultured and observed closely for clinical signs of respiratory disease prior to and during the experimental period. The effectiveness of the vaccine may then be evaluated.

MAJOR FINDINGS: Only a few of the vaccinated and control rabbits were cultured for Pasteurella, Bordetella. Unfortunately we did not find any significant difference in the incidence of Pasteurella - Bordetella isolations in the two groups, nor were there any significant differences in the two groups in clinical observations (snuffles). Therefore, the vaccine was ineffective probably due to (1) Age of vaccination (already harboring bacteria before inoculation); (2)

possibly due to route of administration; and (3) part of study (vaccination) done at Pel-Freez. This study suggests that an "in-house" experiment would be more reliable and the rabbits would be easier to monitor. Recent literature suggests that vaccination of mothers during pregnancy may be useful.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Approximately 70-80% of incoming NZW rabbits harbor Pasteurella multocida and Bordetella bronchiseptica in their nasal passages. Those animals succumbing to respiratory disease (pneumonia, pleuritis, otitis internal) have almost invariably been positive for P. multocida. Antibiotics have been found to be ineffective against rabbit "snuffles"; moreover, antibiotics cannot be used because of the nature of research at NIEHS. Vaccination is acceptable and if effective, can significantly reduce the loss of valuable experimental animals.

PROPOSED COURSE: Study discontinued.

KEYWORD DESCRIPTORS: 1. An autogenous bacterin for rabbit snuffles.
2. Combined Pasteurella - Bordetella bacterin for rabbits.

Project No.: Z01 ES 30028-04 EBCB

1. Environmental Biology and Chemistry Branch
2. Comparative Biology Section
3. Research Triangle Park, N.C.

PHS-NIH

Individual Project Report

July 1, 1974 through June 30, 1975

PROJECT TITLE: "Evaluation of a Captive-Born and Reared Colony of Opossums"

PREVIOUS SERIAL NUMBER: NIEHS-EBC-028-3

PRINCIPAL INVESTIGATOR: Donald B. Feldman, D.V.M.

OTHER INVESTIGATORS: None

COOPERATING UNITS: None

MAN YEARS

Total: 0.2

Professional: 0.1

Other: 0.1

PROJECT DESCRIPTION

OBJECTIVES: To evaluate the physiologic, pathologic and reproductive parameters of a "closed" colony of captive-born opossums.

METHODS EMPLOYED: During the 1974 breeding season (Jan.-May), opossums were bred by 1 of 3 methods: (1) conventional (2 females, 1 male); (2) on the basis of vaginal smears, and (3) during post-lactational estrus (which occurred after sucklings were removed from the pouch). These methods of reproduction were evaluated to determine the most efficient means of obtaining opossum fetuses of known birth dates.

MAJOR FINDINGS: The conception rate from conventional breeding in 1974 was 42% (8 litters from 20 breeding females). Six of 12 opossums (50%) gave birth to young on the basis of sperm positive vaginal smears. Thirteen of 35 females (32%) conceived approximately 10 days after their young were removed from the pouch. Thus far, through 12 weeks of the 1975 breeding season 11 of 30 females (36%) have given birth through conventional breeding.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: This colony has been monitored from year to year as a feasible means of providing sufficient animals for research. During the past year research utilizing the opossum has been considerably reduced and the concomitant demands for neonates has lessened. Because of the value of those opossums, the colony will probably be perpetuated but only a minimal number of breeding opossums will be maintained from year to year. In the event of increased demand, the colony can be expanded.

PROPOSED COURSE: Continued monitoring of the colony.

KEYWORD DESCRIPTORS: Opossum breeding; opossum neonates; post-lactational estrus.

PUBLICATIONS

Feldman, D.B. and Ross, P.W.: Methods for obtaining neonates of known age from the Virginia opossum (Didelphis marsupialis virginiana). (Accepted)
Lab. Anim. Sci.

Project No.: Z01 ES 30029-05 EBCB

1. Environmental Biology and Chemistry Branch
2. Chemistry Section
3. Research Triangle Park, N.C.

PHS-NIH

Individual Project Report

July 1, 1974 through June 30, 1975

PROJECT TITLE: "Prostaglandins as Environmental Factors"

PREVIOUS SERIAL NUMBER: NIEHS-EBC-030-2

PRINCIPAL INVESTIGATORS: Phillip W. Albro, Ph.D.

OTHER INVESTIGATORS: R. Thomas

COOPERATING UNITS: None

MAN YEARS

Total:	0.0
Professional:	0.0
Other:	0.0

PROJECT DESCRIPTION

OBJECTIVES: (1) Development of a highly sensitive, specific analytical procedure for qualitative and quantitative determination of prostaglandins. (2) Evaluation as to whether exogenous prostaglandins should be considered environmental health factors, either relative to ingestion or topical exposure. (3) Determination as to whether endogenous prostaglandins play a significant role in the response of animals to drugs, pesticides, antigens and the like.

METHODS EMPLOYED: Have been described in two previous publications (J. Chromatogr. 44: 443, 1969; Prostaglandins 1: 133, 1972).

MAJOR FINDINGS: This project received no study during the present reporting period.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Having eliminated several other possibilities, we have presently concentrated on the role of prostaglandins in the anaphylactic response as being related to their possible environmental significance and not under saturating study elsewhere.

PROPOSED COURSE: Upjohn has begun to license prostaglandins for drug use in this country. Should reason to believe harmful effects may arise from such use appear in the future, we may consider reinstituting this project on an active basis; however, in view of the higher priorities assigned to other projects, we presently consider this project terminated.

KEYWORD DESCRIPTORS: Prostaglandins; analytical procedures; anaphylaxis, synergism.

ENVIRONMENTAL BIOMETRY BRANCH

ENVIRONMENTAL BIOMETRY BRANCH

Summary Statement

The Environmental Biometry Branch fulfills a dual mission, functioning both in a service capacity to the intramural program, and also as an applied research group. Statistical support for the Institute is provided mainly for the design of experiments and in the analysis of laboratory data. Data processing and computer programming services are available to the research staff of the Institute and are routinely employed by the Environmental Biometry Branch in both its statistical applications and research work.

Research activity is currently being conducted in the general areas of statistical methodology, biomathematics and epidemiology. The statistical research involves both the development of methodology generated from data analysis needs, and the investigation of topics such as clinical trial design and low-dose extrapolation, which are of abiding interest to the NIH. Biomathematics research is primarily collaborative, being focused mainly on the mathematical modeling of biological processes. Finally, the epidemiological work being done is concerned with the statistical analysis of existing data bases, the emphasis being on an attempt to detect environmental components and quantify their implications upon human health.

Statistical and Programming Support

Statistical and/or programming support was provided to virtually every section of the various branches of the intramural program as well as to the office of the Director during the past twelve-month period. While the bulk of the statistical service effort would still be classified under the general heading of data analysis, there was a definite increase in the degree to which various members of the Branch participated in the initial design aspects of a number of intramural research programs. Particular emphasis was placed on the assessment of design feasibility and the coordination of study objectives with the level of institute resources available for the performance of the experiment in question.

In addition to an increase in the overall volume of statistical analysis support requested from the Branch throughout the preceding year, there has also been an increase in the degree of complexity of many of the problems encountered in the consulting operation. An optimization procedure based on response surface techniques was utilized to estimate the best combination of a host of experimental parameters to maximize *in vitro* development of rabbit morulla. A review of the existing literature on the statistical aspects of protein receptor analysis indicated that even the most sophisticated approaches currently in use do not properly account for the true error structure of the data. Experimental observations are usually forced to conform to traditional regression techniques which ignore variability in one of the measured responses. Generalized non-linear regression techniques, which allow for appropriate

weighting of both dependent and independent variables, are being investigated and will, hopefully, provide a more rigorous solution to the problem. Analysis of data generated from a study designed to assess the synergistic potential of two environmental agents has raised the question of the most appropriate means of interfacing the pharmacological definition and the statistical analysis of synergism or interaction. This question is of particular importance when dealing with a zero-one response or frequency-type data, when the usual concept of additivity may no longer be applicable. Finally, a problem in population screening for genetic variability has instigated an investigation of methods of combining the results from multidimensional contingency tables of varying sample size and, in some instances, varying dimensions.

The Branch has continued its policy of employing nonparametric procedures in analyzing laboratory data whenever it is feasible to do so, since the small sample sizes associated with many of the experiments conducted at the Institute preclude the evaluation of the appropriateness of the assumptions underlying most parametric techniques.

As is the case with statistical consulting and data analysis, programming support for the Institute has been expressed in a wide variety of forms. The Branch has been instrumental in implementing and maintaining programming support for two computer based inventory systems. The first system is an inventory management system for the warehouse, while the second is concerned with a periodically updated listing of all radioactive isotopes held at the NIEHS. On the basic research level, ongoing support is being provided to the Developmental Toxicology, Aerotoxicology, Pharmacokinetics, and Population Genetics Sections of the Institute's intramural program through the development and systematic revision of computerized packages for performing protein receptor analysis, chronic study surveillance, pharmacokinetic modelling, and gene frequency deviation detection, respectively. In addition, individual scientists interested in accessing the teratogenic potential of a spectrum of environmental agents have been assisted in their endeavors by the implementation of a highly flexible computerized system for statistical analysis. Finally, a historical file of carcinogenic and mutagenic screening studies readily amenable to various cross-referencing and searching techniques is being made available. The computer is also used extensively for simulation studies, within the Branch as well as for other Branches.

Research Activities

Epidemiology

The investigation of potential association between various environmental factors and the incidence of anencephalic births in North Carolina was completed. State data failed to confirm Renwick's hypothesis that the onset of anencephalus is related to the exposure to some undefined substance in blighted potatoes. In addition, urban-rural differences in the proportion of estimated anencephalic conceptions occurring in various months and seasons showed no marked association with pesticide usage.

A collaborative effort with graduate students and faculty from the University of North Carolina, Department of Epidemiology, has been inaugurated. A major

goal will be to attempt to extend relevant findings from the Institute's intramural research program into the human setting. At the current time two projects are in the feasibility assessment stage. One of the proposals is concerned with the potential health hazard associated with trace or low levels of known or suspected carcinogens and/or mutagens in public drinking water sources. This same topic is being independently studied within the Branch.

Mathematical Biology

In collaboration with the Pharmacology Branch research efforts in pharmacokinetics were concentrated in two areas: 1) investigation of the dynamic interaction between the lung and circulating chemicals using the isolated perfused lung system and 2) pharmacokinetic studies of several polychlorinated biphenyls (PCB's). Detailed models for the dynamic interaction between the lung and several basic amines have been developed. It has been shown that these amines not only accumulate, i.e. high tissue to blood concentration ratios, but they do persist in lung tissue. It has also been demonstrated that the lung has a transport system for the removal of certain prostaglandins from the circulation. The distribution, storage, metabolism, and excretion of four PCB's were studied in the rat. The hexachloro PCB studied is stored in fat and skin with an extremely long half-life. Pharmacokinetic models are being developed for these compounds.

In collaboration with the Environmental Mutagenesis Branch mathematical models are being studied which describe the effect of migration upon gene frequencies. Specifically, branching processes are being used to determine how far a unique neutral gene will migrate before it disappears from the population and to determine the geographical distribution of gene frequencies.

Risk Assessment

The problem of human risk estimation based on laboratory data is being studied by the Environmental Biometry Branch. Specific attention is given to carcinogenesis and the methods used for estimating human risk, at low environmental exposure levels. Considerations of mechanism such as hit models and multistage models are being compared. Also, the importance of background incidence and induction time are being evaluated. These considerations have led to the investigation of optimal experimental designs.

In the area of carcinogenic screening some inexpensive presumptive tests are being examined. In particular, the use of mutagenic tests as predictors of carcinogenesis is being studied from a decision-theoretic viewpoint.

Project No.: Z01 ES 40001-05 EBB
1. Environmental Biometry Branch
2.
3. Research Triangle Park, N.C.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

PROJECT TITLE: "Clinical Trials and Statistical Selection Procedures"

PREVIOUS PROJECT NUMBERS: NIEHS-B-001; NIEHS-B-005

PRINCIPAL INVESTIGATORS: D. G. Hoel, Ph.D., R. Simon, Ph.D. (NCI), and
G. H. Weiss, Ph.D. (DCRT)

OTHER INVESTIGATORS: None

COOPERATING UNITS: None

MAN YEARS

Total:	0.2
Professional:	0.2
Other:	0.0

PROJECT DESCRIPTION

OBJECTIVES: To develop efficient experimental designs for selecting the best of several experimental treatments.

METHODS EMPLOYED: Data from controlled laboratory or clinical trials are assumed to occur sequentially in time where the individual responses are dichotomous. Sequential regions have been developed for deciding when to terminate the experiment and which treatment to select as most effective.

MAJOR FINDINGS: Improved likelihood stopping procedures have been developed which reduce the total number of patients in the clinical trial. Techniques for the inclusion of concomitant information such as patient age differences, differing clinical centers, etc., have been produced.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The emphasis of these sequential designs is to minimize the number of trials on inferior treatments. This has been overlooked in classical clinical trials and is a critical concern when dealing with human populations.

PROPOSED COURSE: The clinical trial designs which take into account concomitant information will undergo continued study and development.

KEYWORD DESCRIPTORS: Clinical trial design, sequential procedures,
adaptive sampling

PUBLICATIONS

Hoel, D. G., and Weiss, G. H.: A clinical trial design with a fixed maximum number of failures. Communications in Statistics (in press).

Simon, R., Weiss, G. H., and Hoel, D. G.: Sequential analysis of binomial clinical trials. Biometrika (in press).

Project No.: Z01 ES 40002-05 EBB
1. Environmental Biometry Branch
2.
3. Research Triangle Park, N.C.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

PROJECT TITLE: "Statistical Methodology and Modeling in the Analysis of Teratology Data"

PREVIOUS PROJECT NUMBER: NIEHS-B-004

PRINCIPAL INVESTIGATOR: J. K. Haseman, Ph.D.

OTHER INVESTIGATORS: M. D. Hogan, Ph.D., F. O. Lin, Ph.D., and
R. E. Staples, Ph.D. (Environmental Toxicology Branch)

COOPERATING UNITS: None

MAN YEARS

Total:	0.8
Professional:	0.8
Other:	0.0

PROJECT DESCRIPTION

OBJECTIVES: To develop statistical test procedures appropriate for the analysis of teratology data and to devise a computerized system for the implementation of these techniques.

METHODS EMPLOYED: Computer simulation studies have been carried out to determine the actual significance level and power associated with a number of statistical tests frequently employed in the analysis of teratology data. Modifications of several nonparametric procedures (Mann-Whitney U test, Jonckheere's test) have been derived that take into account ties at a single extreme value, a situation that frequently arises in teratology studies. Computer programs incorporating these techniques are being developed to facilitate the processing of data from teratology experiments.

MAJOR FINDINGS: When litter effects are present statistical test procedures that treat the individual fetus as the basic experimental unit may have actual significance levels up to 20 times the nominal level. Even in the unlikely event that there are no litter effects there appears to be little loss in sensitivity in adopting the litter rather than the fetus as the basic sampling unit.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE:

Teratology experiments constitute an important part of the overall NIEHS intramural program. Thus it is essential to develop appropriate statistical methodology and data processing capabilities to handle the large volume of data generated by studies in this area of research.

PROPOSED COURSE: Conduct further statistical research in the design and analysis of teratology studies, and continue the development of a computerized system for the processing of teratology data.

KEYWORD DESCRIPTORS: Teratology, litter effect, nonparametric statistics, computer simulation, data processing, experimental unit

PUBLICATIONS

Haseman, J.K. and Hoel, D.G.: Tables of Gehan's generalized Wilcoxon test with fixed point censoring. J. Stat. Comp. Simulation 3: 117-135, 1974.

Staples, R.E. and Haseman, J.K.: Selection of the appropriate experimental units in teratology. Teratology 9: 259-260, 1974.

Project No.: Z01 ES 40003-04 EBB
1. Environmental Biometry Branch
2.
3. Research Triangle Park, N.C.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

PROJECT TITLE: "Nonparametric Tests in Biometry"

PREVIOUS PROJECT NUMBERS: NIEHS-B-007; NIEHS-B-014

PRINCIPAL INVESTIGATORS: K. S. Crump, Ph.D., and F. O. Lin, Ph.D.

OTHER INVESTIGATORS: None

COOPERATING UNITS: None

MAN YEARS

Total:	0.3
Professional:	0.3
Other:	0.0

PROJECT DESCRIPTION

OBJECTIVES: To develop statistical tests for interaction between two treatments, and to study the range of applicability of the tests.

METHODS EMPLOYED: Monte-Carlo techniques were used to compare several tests for interaction.

MAJOR FINDINGS: A nonparametric test for interaction which had been previously reported in the literature was found to be seriously affected by the presence of first-order effects in the data. The first order effects tend to mask out the interactive effect, and the test for interaction may yield a negative result when in fact a significant interaction between the two treatments is present. A modification of the test was developed that mitigated this difficulty. The modified test was shown to have meritorious power relative to other nonparametric tests for interaction.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The determination of whether two drugs or treatments have a synergistic effect often arises in environmental biology. It is important to know if two drugs applied together elicit a response which is significantly different from the superposition of the two responses obtained when the drugs are administered separately.

PROPOSED COURSE: The project will be terminated.

KEYWORD DESCRIPTORS: Nonparametric, interaction, Monte-Carlo

Project No.: Z01 ES 41001-01 EBB
1. Environmental Biometry Branch
2.
3. Research Triangle Park, N.C.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

PROJECT TITLE: "Low Dose Risk Estimation from Experimental Carcinogenesis"

PREVIOUS PROJECT NUMBER: None

PRINCIPAL INVESTIGATORS: D. G. Hoel, Ph.D., K. S. Crump, Ph.D., and
C. H. Langley, Ph.D. (Environmental Mutagenesis
Branch)

OTHER INVESTIGATORS: C. A. Barnes (University of North Carolina)

COOPERATING UNIT: Population Genetics and Epidemiology Section, Environmental
Mutagenesis Branch

MAN YEARS

Total: 1.2
Professional: 1.0
Other: 0.2

PROJECT DESCRIPTION

OBJECTIVES: In order to estimate human risk based on animal carcinogenesis experiments it is currently necessary to extrapolate data from high dose experiments to low doses. The purpose of this investigation is to study the various mathematical and probabilistic methods for extrapolation with particular emphasis on those which attempt to relate mechanistically to the carcinogenic process.

METHODS EMPLOYED: Various possible models of carcinogenesis were analyzed with respect to low dose kinetics. Considerations of mechanism such as hit models and multistage models were compared to so-called log normal response models. The importance of background incidence and induction time were evaluated.

These considerations led to the investigation of optimal experimental design considerations. Specifically, the sample size and dose rate were optimized.

MAJOR FINDINGS: 1) If carcinogenesis by an external agent is considered to be augmenting an already on-going spontaneous process, then the response will be linear at low dose. 2) If the origin of a cancer is an event in a single cell (as distinct from a whole tissue) then the low dose response will be linear. 3) In general, time of cancer induction must be heavily dependent on dose rate for such consideration to play a role in low dose risk. 4) Linear

extrapolation is not always the conservative technique it is thought to be. 5) The optimal experimental dose for obtaining the maximal acceptable dose is robust with respect to models when one is extrapolating linearly. 6) Little is gained by large sample sizes in experiments aimed at low dose risk estimation.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: In order to determine risk associated with possible or actual human exposure of carcinogens it is usually necessary to determine the carcinogenicity of the agent in experimental laboratory animals. The observed risk is then extrapolated down to human exposure levels and then converted to human risk by some conversion factor. The particular model chosen to extrapolate the experimental risk to the low dose risk is critical in determining the order of magnitude of the exposure. This research seeks to investigate the various aspects of this procedure.

PROPOSED COURSE: 1) To modify the hit models to allow a hit cell to be repaired by some stochastic mechanism and determine if repair can have any significant qualitative effect upon the dose response curve. 2) To consider the implication of variance among individuals in sensitivities to an insult of a carcinogen upon low dose risk estimation.

KEYWORD DESCRIPTORS: Carcinogenesis, low dose, extrapolation, risk estimation, models

Project No.: Z01 ES 42001-03 EBB
1. Environmental Biometry Branch
2.
3. Research Triangle Park, N.C.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

PROJECT TITLE: "Studies on Pharmacokinetic Functions of the Lung"

PREVIOUS PROJECT NUMBERS: NIEHS-B-008; NIEHS-B-018

PRINCIPAL INVESTIGATORS: M. W. Anderson, Ph.D., and T. E. Eling, Ph.D.
(Pharmacology Branch)

OTHER INVESTIGATORS: R. J. Sensenig, B.S., and T. A. Clemmer, M.A.

COOPERATING UNITS: Pharmacology Branch

MAN YEARS

Total:	1.3
Professional:	1.2
Other:	0.1

PROJECT DESCRIPTION

OBJECTIVES: The basic objective is to understand the mechanisms which control the uptake, accumulation, and metabolism of chemicals in lung tissue.

METHODS EMPLOYED: An isolated perfused lung system was used to obtain kinetic data for the uptake and accumulation of chemicals by the lung and to study the metabolism of chemicals by the lung.

MAJOR FINDINGS: 1) An isolated perfused lung system was developed so that initial rates of uptake and efflux of accumulated chemicals could be investigated. Using this system, the dynamic interaction between the lung and several basic amines has been examined. Our studies show that some exogenous basic amines not only accumulate in lung tissue, i.e. high tissue to blood concentration ratios, but they also persist in lung tissue. By persistence we mean that if the amine is allowed to accumulate in lung tissue, then some of it cannot be effluxed from the lung. This noneffluxable component is not due to covalent binding but appears to be a compartmentalization. Of the basic amines which we have examined in detail, imipramine, methadone, and amphetamine, only the latter does not persist. A transport system is not involved in the removal of imipramine and methadone from the circulation by the lung. A transport system is involved in the removal of amphetamine, and this transport system is probably the nonadrenaline transport system which exists in lung tissue. The high tissue to blood concentration ratios, >300 for imipramine and methadone, are due to several types of binding sites that exist for these

compounds in lung tissue. We think that the persistence of the amines could be related to their toxicity and in particular to the phospholipidosis observed in lung tissue after chronic treatment of animals with these compounds. We are pursuing the persistence aspect of basic amine interaction with lung tissue in both our isolated perfused lung system and with whole animal studies. We are also developing physiological models to describe the dynamic interaction of these compounds with lung tissue.

2) The lung has the ability to remove from the circulation and subsequently modify many endogenous chemicals, e.i. 5-HT, bradykinin, angiotensin, and the prostaglandins. We have studied the removal and modification of 5-HT and the prostaglandins in detail. Although it has been previously demonstrated that 5-HT is deactivated by the lung, the system has permitted the first characterization of the dynamic aspects of the transport and the metabolic component of this system and of the interactions between them. The vasoactive hormone is removed from the circulation by a uni-transport system, metabolized by the intracellular monoamine oxidase to 5-hydroxy indole acetic acid and then the metabolite effluxes by diffusion back into the circulation. We have shown the existence of a transport system in lung tissue for the removal of some prostaglandins from the circulation. After their removal they are converted to the biologically inactive 15-keto metabolite by means of prostaglandin dehydrogenase. This removal of prostaglandins by lung is specific since PGA and PGB are not removed, whereas PGF_{2α} and PGE₁ are removed. We are further examining this transport system for prostaglandins. We are also examining the effects that other chemicals which accumulate in lung have on these inactivation systems and the effects that exposure to environmental pollutants (ozone, NO₂, SO₂, etc.) have on these systems.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Refer to Z01 ES 80008-01 PB, Z01 ES 80009-04 PB, and Z01 ES 80010-04 PB, which are projects of the Pharmacology Branch.

PROPOSED COURSE: Refer to Z01 ES 80008-01 PB, Z01 ES 80009-04 PB, and Z01 ES 80010-04 PB, which are projects of the Pharmacology Branch.

KEYWORD DESCRIPTORS: Lung pharmacokinetics, lung, basic amines, prostaglandins

PUBLICATIONS

Anderson, M. W., Eling, T. E., and Philpot, R. M.: Metabolic functions of the lung. Monograph 5 of the Series "Lung Biology in Health and Disease". in Lenfant, C. (Ed.): Steriods and Drugs, Chapter 5. New York, Marcel Dekker, Inc. (in press)

Anderson, M. W., Pickett, R., Orton, T. C., Eling, T. E.: Accumulation of amines in isolated perfused rabbit lung. J. Pharmacol. Exptl. Therap. 189(2): 456-466, 1974.

Eling, T. E., Pickett, R. D., Orton, T. C., and Anderson, M. W.: A study of the dynamics of imipramine accumulation in the isolated perfused rabbit lung. Drug Metabolism and Disposition (in press)

Orton, T. C., Anderson, M. W., Eling, T. E., Fouts, J. R.: Xenobiotic accumulation and metabolism by isolated perfused rabbit lungs. J. Pharmacol. Exptl. Therap. 186(3): 482-497, 1973.

Pickett, R. D., Anderson, M. W., Orton, T. C., and Eling, T. E.: The pharmacodynamics of 5-hydroxy tryptamine uptake and metabolism by the isolated perfused rabbit lung. J. Pharmacol. Exptl. Therap. (in press)

Project No.: Z01 ES 42002-03 EBB
1. Environmental Biometry Branch
2.
3. Research Triangle Park, N. C.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

PROJECT TITLE: "Pharmacokinetic Studies of Polychlorinated Biphenyls"

PREVIOUS PROJECT NUMBERS: NIEHS-B-008; NIEHS-B-018

PRINCIPAL INVESTIGATORS: M. W. Anderson, Ph.D., T. E. Eling, Ph.D.
(Pharmacology Branch), and H. B. Matthews, Ph.D.
(Pharmacology Branch)

OTHER INVESTIGATORS: R. J. Sensenig, B.S., and T. A. Clemmer, M.A.

COOPERATING UNITS: Pharmacology Branch

MAN YEARS

Total:	1.4
Professional:	1.3
Other:	0.1

PROJECT DESCRIPTION

OBJECTIVES: The basic objective is to understand the mechanisms which control the distribution, storage, metabolism, and excretion of lipophilic chemicals, such as polychlorinated biphenyls, in various species.

METHODS EMPLOYED: The approach used by R. L. Dedrick and D. S. Zaharbo in the development of pharmacokinetic models for several drugs is being employed to develop similar type models for the PCB's.

MAJOR FINDINGS: Information gained from pharmacokinetic investigations with various drugs has been helpful for a better understanding of their pharmacology and for their more efficient use. Similar studies of environmental contaminants should lead to a better understanding of the potential hazards associated with these compounds and more accurate predictions of residue levels. However, extrapolation of the techniques and data used in the study of hydrophilic and readily metabolized drugs to the study of hydrophobic and relatively slowly metabolized environmental contaminants such as chlorinated pesticides and polychlorinated biphenyls (PCB's) has proved difficult. Thus, the number of such studies has been limited and have dealt almost exclusively with the chlorinated insecticide, dieldrin. Nevertheless, a thorough pharmacokinetic study is one of the best methods available which will allow an accurate prediction of the biological accumulation of certain xenobiotic residues currently being reported in the environment.

The distribution and excretion of four polychlorinated biphenyls (PCB), 4-chloro-, 4,4'-dichloro-, 2,4,5,2',5'-pentachloro and 2,4,5,2',4',5'-hexachlorobiphenyl, were studied in the rat. The concentration of each of these PCB's was determined in major organs and tissues at times varying from 15 min up to 42 days after an intravenous injection of a single 0.6 mg/kg dose. The relative amounts of each PCB and its metabolites in each major tissue were determined at selected time points.

Some general comments regarding the distribution, metabolism, and excretion of PCB's can be deduced from the data obtained for the four PCB's studied in this investigation. First, following their i.v. administration each of the PCB's was rapidly removed from the blood and initially stored primarily in liver and muscle. The relatively large percent of the total dose stored in muscle was due to its large mass, 50% of the body weight, whereas storage in liver resulted from a higher tissue affinity. Second, redistribution of PCB's from liver and muscle to skin and adipose tissue was basically one of redistribution to tissues of lower perfusion and higher affinity. The extremely long half-life of 6-CB in skin was of particular interest since certain skin disorders are symptomatic of intoxication by the more highly chlorinated commercial PCB formulations, of which this compound is a major constituent. Pure 6-CB has also been shown to be acnegenic. Third, the percent of the total radioactivity in each tissue accounted for by the parent PCB's varied with the tissue assayed and decreased as the chlorination of the administered PCB increased. Thus, the kinetics for the removal of radioactivity from the various tissues was more often a measure of the removal of a mixture of compounds than for a single compound. Nevertheless, the rates of removal of total radioactivity were such that they implied the action of first-order processes and that the presence of the two or more types of compounds did not interfere with the removal of one another. The kinetics for the removal of PCB metabolites from tissues may actually be more important to the toxicology of these compounds than the kinetics for the removal of the parent compound, since Yoshimura and Yamamoto have shown that the major metabolite of tetrachlorobiphenyl is five times more toxic than the parent compound. And fourth, metabolism is obviously a key factor in the distribution and excretion of PCB's, since studies in our laboratory indicate that less than 10% of the total dose of these compounds is excreted unmetabolized. Metabolism was, in turn, greatly affected by the degree of chlorination. However, it is not only a simple matter of the number of chlorine atoms on the biphenyl molecule which controls the rate of metabolism, but also the position of the chlorine atoms on the biphenyl molecule. It appears that two adjacent unsubstituted carbon atoms are necessary for an appreciable rate of metabolism of the PCB's. The importance of metabolism and, consequently, two adjacent carbon atoms is seen in the work presented here where more than 90% of the 5-CB dose was excreted in 42 days, whereas the addition of a single chlorine atom at the 4' position to form 6-CB, which does not have two adjacent unchlorinated carbon atoms, confounded the metabolism of this molecule such that extrapolation of the excretion data to infinite time indicates that less than 20% of the total 6-CB dose would ever be excreted by an animal receiving a normal diet. It has been shown that 6-CB is the most prevalent PCB in human tissue.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Refer to Z01 ES 80014-02 PB, Z01 ES 80015-02 PB, and Z01 ES 80016-02 PB, which are projects of the Pharmacology Branch.

PROPOSED COURSE: Refer to Z01 ES 80014-02 PB, Z01 ES 80015-02 PB, and Z01 ES 80016-02 PB, which are projects of the Pharmacology Branch.

KEYWORD DESCRIPTORS: Pharmacokinetic, polychlorinated biphenyls, lipophilic

PUBLICATIONS

Matthews, H. B. and Anderson, M. W.: The distribution and excretion of 2,4,5,2',5'-pentachlorobiphenyl in the rat. Drug Metabolism and Disposition (in press)

Matthews, H. B., Chen, P. R., Mehendale, H. M. and Anderson, M. W.: The metabolism, storage, and excretion of highly chlorinated compounds by mammals. in Kohn, G. K. (Ed.) Mechanism of Pesticide Action. American Chemical Society Symposium Series, American Chemical Society, Washington, D. C., 1974, pp. 54-68.

Project No.: Z01 ES 43001-03 EBB
1. Environmental Biometry Branch
2.
3. Research Triangle Park, N.C.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

PROJECT TITLE: "Epidemiological Investigations of Potential Human Health Hazards"

PREVIOUS PROJECT NUMBERS: NIEHS-B-006; NIEHS-B-017

PRINCIPAL INVESTIGATOR: M. D. Hogan, Ph.D.

OTHER INVESTIGATORS: None

COOPERATING UNITS: Environmental Toxicology Branch and Epidemiology
Department of UNC School of Public Health

MAN YEARS

Total:	0.2
Professional:	0.2
Other:	0.0

PROJECT DESCRIPTION

OBJECTIVES: To identify and/or confirm the presence of various potential hazards in the environment, and (whenever possible) to integrate epidemiological investigations with the laboratory experiments of the basic biologist. Particular emphasis will be given to the extension of relevant findings from the Institute's intramural research program into the human setting.

METHODS EMPLOYED: A collaborative effort focusing on the generation of thesis topic proposals and/or the provision of statistical support with respect to study design and analysis has been inaugurated with Ph.D. candidates in the UNC Department of Epidemiology. Two topics are currently in the feasibility assessment phase. One involves the verification in a human population of certain developmental abnormalities seen in the NIEHS animal experiments following an in utero exposure to early pregnancy hormonal therapy. The second study is attempting to replicate (or refute) the apparent association between sources of drinking water and the incidence of certain types of cancer in New Orleans, but in a different location. In addition to these collaborative efforts a number of statistically oriented projects, such as the development of a computerized package to simulate various types of epidemiological investigations to determine their degree of feasibility, are being explored within the Branch.

MAJOR FINDINGS: The previously reported investigation of anencephaly in the state of North Carolina has been completed. Renwick's hypothesis concerning the association of a toxin in blighted potatoes and anencephaly could not be replicated. Furthermore, urban-rural differences in the proportion of anencephalic deaths occurring in various months and seasons showed no marked association with pesticide usage.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Because of the lengthy lag time that often exists between initial exposure to an environmental agent and the eventual clinical manifestation of chronic disease or a mutagenic effect, it will often be necessary to rely on animal model(s) for the identification of potential human health hazards. Thus, any attempt to verify these models in the human setting are of obvious significance.

PROPOSED COURSE: To be continued.

KEYWORD DESCRIPTORS: Anencephaly, cluster analysis, Renwick's hypothesis, simulation, correlation of animal and human studies.

Project No.: Z01 ES 44001-01 EBB
1. Environmental Biometry Branch
2.
3. Research Triangle Park, N.C.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

PROJECT TITLE: "Mathematical Models for Describing the Effect of Migration upon Gene Frequencies"

PREVIOUS PROJECT NUMBERS: None

PRINCIPAL INVESTIGATORS: K. S. Crump, Ph.D., and J. H. Gillespie, Ph.D.
(Environmental Mutagenesis Branch)

OTHER INVESTIGATORS: None

COOPERATING UNITS: Population Genetics and Epidemiology Section,
Environmental Mutagenesis Branch

MAN YEARS

Total:	0.6
Professional:	0.5
Other:	0.1

PROJECT DESCRIPTION

OBJECTIVES: 1) To construct probabilistic models for determining how far a unique neutral gene will migrate before it disappears from the population and 2) to determine the geographical distribution of gene frequencies given that the gene is still present in the population.

METHODS EMPLOYED: Branching process models, both discrete and continuous time, were used to describe a population experiencing birth, death, and migration. Migration is assumed to take place in one or two dimensions.

MAJOR FINDINGS: Mathematical expressions have been derived for the mean and variance-covariance of the numbers of individuals carrying the particular gene that ever appear at various distances. Expressions have also been derived for the correlation, after a long time has elapsed, between the numbers of individuals carrying the gene that are present in two localities separated by various distances.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: An important component in variance in individual responses to environmental insults may be genetic variation. Through this project we are attempting to answer some basic questions about the role of the geographic distribution of populations and migration upon the establishment and maintenance of genetic variability.

PROPOSED COURSE: To consider the predictions of the models when applied to real populations and to compare these models with those of other investigators.

KEYWORD DESCRIPTORS: Population genetics, migration, branching processes, neutral gene

ENVIRONMENTAL BIOPHYSICS BRANCH

ENVIRONMENTAL BIOPHYSICS BRANCH Summary Statement

Major research efforts of the Environmental Biophysics Branch during fiscal year 1975 were: (1) developing a better understanding of the mechanisms of the temporary and permanent shift of the threshold of hearing following exposure to noise and/or other ototoxic agents; (2) developing insights on some of the non-auditory effects of noise exposure; (3) developing nonionizing radiation exposure systems, dosimetry techniques, and determination of non-thermal and thermal effects of nonionizing radiation on biological systems, and; (4) developing theoretical bases for predicting dose-response relationships and experimentally testing models at low dose and low dose rates at the cellular level using ionizing radiation.

Noise

The noise research conducted and supported by the NIEHS is an integral component of its overall effort to determine what factors in the environment, operating singly or in combination, adversely affect man's health. The general objectives of the NIEHS noise effects program are: to increase understanding of physiological, biochemical, and electroneural mechanisms, the disruption of which by noise and other ototoxic agents leads to the cellular and neural damage which characterizes permanent hearing loss; to identify various environmental chemicals, drugs, and other agents which potentiate the hearing loss from noise and to characterize the degree and extent of additional loss to be expected; and to investigate specific endocrine, immunologic, physiologic, pharmacologic, teratogenic, cardiovascular, and other mechanisms which may be affected by chronic noise exposure.

Studies have been initiated to explore the location and nature of physiological changes underlying the temporary threshold shift of hearing. The cochlear microphonic, whole nerve action potential and endocochlear potential of noise exposed guinea pigs were examined to elicit the hearing recovery process. Measuring these electric potentials produced by pure test tones following noise exposures at various sound pressure levels showed very evident hearing threshold shifts at the lower test tones and higher noise exposures with subsequent recovery. Prolonged noise exposure would have resulted in a permanent threshold shift in hearing. Evidence gained from the temporary threshold shift data will be useful for predicting a permanent hearing loss over an extended exposure time. The next step will be to measure the temporary threshold shift caused by exposure to ototoxic antibiotics and in combination with noise.

Studies are also underway to confirm the ototoxicity of methyl mercury and relate this to the resulting concentrations of methyl mercury found in the cochlear fluids.

A piezoelectric transducer has been developed which when in direct contact with the ossicular chain can impart highly defined signals into the cochlea. This device avoids the necessity for complex microphonic measurements of sound pressure levels at the tympanic membrane. Concurrently under development is a double beam laser microscope for measuring displacement and waveform of the ossicular chain and basilar membrane.

Acute noise exposure has been shown to markedly affect various hormone secretions. Immunological function can be affected by altered levels of certain hormones. Studies are being conducted to detect suppression or stimulation of cell mediated immunity in noise exposed rodents. Early data shows some suppression of lymphocyte production following noise exposure.

Nonionizing Radiation

The general objectives of the microwave research activities are: to develop additional microwave exposure techniques with continuous wave, pulsed wave and modulated wave delivery modes and known field characteristics; to develop dosimetric techniques (both instrumental and analytical); and to design and build a system to evaluate the electrical properties of biological material; to investigate the biophysical mechanisms of interaction of CW, pulsed and modulated microwave radiation of frequencies 1 to 10 GHz on biological material at the cellular and macromolecular levels by (a) exposing various bacteria and bacteriophage systems and (b) exposing mammalian red and white blood cells; to evaluate the effects of CW and pulsed 2.45 GHz microwave radiation on embryonic development and reproduction by (a) exposing the developing Japanese quail embryos to various levels of microwave radiation throughout the gestation period; (b) exposing pregnant mice throughout the gestation period to low level (10 mW/cm^2) radiation; (c) determining the effects of reproduction of the quail and mice obtained in (a) and (b) by maintaining the offspring to sexual maturity and mating them; to study the effects of CW and pulsed 2.45 GHz microwave radiation on the development of the immunological response by testing the quail and mice exposed during development for resistance to infection and antibody production; to investigate the effects of CW, pulsed and modulated microwave radiation at frequencies of 1 to 10 GHz on the functioning of the central nervous system and behavior by; (a) determining the effects on stimulus time-duration thresholds for producing an action potential and on the conduction velocity of the action potential along the nerve; (b) studying the effects of the radiation on reflex response and synaptic transmission in the cat, and; (c) supporting contract research on the effects of long-term (chronic), low level microwave exposure on behavior, EEG and the neuroendocrine system.

During the past year, the research program of NIEHS has concentrated on determining nonthermal effects of microwave radiation at 2450 MHz. Studies on the effects of microwave radiation on cell systems, embryo development and neural response have been the major efforts. Experiments on DNA denaturation, bacteria replication, and bacteria-bacteriophage systems have been conducted. Maintaining the biological systems at their viable temperature during exposure have resulted in no change in the exposed as compared to the control systems for the end points investigated. Exposure of rabbit and human erythrocytes and lymphocytes in vitro are in progress.

Embryo developmental effects have been studied using the developing Japanese quail embryo as the test system. Exposures of the embryo for four hours each of the first five days of development and 24 hours on the second day of development have not produced any gross deformities. The hatched birds from the groups exposed on day two of development have slightly lower hemoglobin levels. Experiments to expose the developing embryo throughout the entire development period (14 days) are now in progress. No hatching has occurred in the groups exposed to 85-120 mW/cm² using 2450 MHz radiation even though the embryos were maintained at or near incubating temperature (37°C). Exposure levels will be decreased until hatching does occur.

Studies on the effects of 2450 MHz microwave radiation on neural function were begun during the year. Some research has been performed in determining the effects of exposure of the spinal cord of cats on the reflex response and synaptic transmission. The exposure power density level was 10 mW/cm². Preliminary results show a small but statistically significant increase in the magnitude of the neural response. These studies will be continued at higher exposure intensities to determine if the effect increases with an increase in exposure levels.

Research is needed to determine biological effects resulting from low level chronic exposures for both CW and EMP radiation. Very little work is presently being performed at these conditions due to the time and expense required. Until these results are obtained, the potential hazards of nonionizing radiation will continue to be an unanswered question regardless of the results from short term experiments.

Ionizing Radiation

Theoretical-mathematical modeling of the effects of ionizing radiation at the cellular level has been performed. The theoretical model agrees with experimental cell survival curves available in the literature. Experimental work is needed to specifically test the model under low dose, low dose rate conditions. Such work is currently being considered through contract mechanisms.

Development of respiratory tract deposition models in hamsters, rats, beagle dogs and man are being performed by contract. Work includes development of theoretical deposition models, construction of physical models and field evaluation of the physical-theoretical models. This phase of the contract work is expected to be completed in fiscal year 76.

Project No.: Z01 ES 50001-01 EBpB
1. Environmental Biophysics Branch
2.
3. Research Triangle Park, N. C.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

PROJECT TITLE: "Development of a Distortion Compensating Driver for Condenser Earphones"

PREVIOUS SERIAL NUMBER: None

PRINCIPAL INVESTIGATOR: Teruzo Konishi, M.D., Ph.D., Reginald O. Cook,
Walter Friauf

OTHER INVESTIGATORS: None

COOPERATING UNIT: Biomedical Engineering and Instrumentation Branch, NIH

MAN YEARS

Total:	.05
Professional:	.04
Other:	.01

PROJECT DESCRIPTION

OBJECTIVES: Overall objective was to develop a driver for a condenser microphone which counteracts the 12.5% or more harmonic distortion ordinarily present from the output waveform when condenser microphones are used in drivers.

METHODS EMPLOYED: Using mathematical techniques and electrical engineering theories a circuit was designed which counteracts the harmonic distortion normally present in condenser microphone outputs. Tests of the output of the electrical signal of the driver and of the acoustic signal and harmonic distortion present when driving condenser microphones were undertaken with the aid of volt meters, wave analyzers, real time analyzers and other complicated electrical and sound analysis equipment.

MAJOR FINDINGS: The electrical output from the distortion compensating driver is linearly proportional to the square root of the input signal. The maximal acoustical output level was approximately 120 dB SPL in a closed rigid cavity (2 cc coupler) for 1" B & K condenser microphone. The driver developed by us reduced the second harmonic distortion from 20 to 40 dB at the maximal output.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE:
Surprising as it may seem, it is a very difficult thing to build a system which will maintain a desired SPL across frequency and produce the desired wave shape

at the tympanic membrane. Complex sounds are needed to elicit the inner relationships of mechanical/physio-biochemical system which the cochlea is. The method most used in producing and maintaining control of the wave shape has involved the insertion of some kind of acoustic generator and diaphragm into a coupler which is inserted into the ear canal, and the now totally enclosed air column between diaphragm and tympanic membrane driven acoustically. Use of this method has been beset with the production of distortion products at the high sound pressure levels to which the couplers must be driven to over stimulate the ear. Since many auditory physiology/noise effects experiments are designed around looking for the presence of driver created distortion products in the acoustic signal is intolerable.

PROPOSED COURSE: Maximum harmonic distortion in the acoustic signal is measured in the external ear canal of guinea pigs or cats. The overall electrical noise level at the output terminals of the amplifier is measured. A report of the successful development of this transducer using solid state components will be submitted as a letter to the Editor of the Acoustical Society of America as many laboratories have need for such driver conditioning circuits.

KEYWORD DESCRIPTORS: Condenser microphone, distortion compensating driver for condenser earphones.

Project No.: Z01 ES 50002-02 EBpB
1. Environmental Biophysics Branch
2.
3. Research Triangle Park, N. C.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

PROJECT TITLE: "Ototoxicity of Methyl Mercury"

PREVIOUS SERIAL NUMBER: NIEHS-EB-007

PRINCIPAL INVESTIGATOR: Teruzo Konishi, M.D., Ph.D.

OTHER INVESTIGATORS: Philip E. Hamrick, PH.D, Phillip J. Walsh, Ph.D.
Alexandra Yankwich, M.S.

COOPERATING UNIT: None

MAN YEARS

Total	0.4
Professional	0.3
Others	0.1

PROJECT DESCRIPTION

OBJECTIVES: The objectives of this project are to study the effect of methyl mercury on cochlear potentials and to determine the concentration of methyl mercury in the cochlear fluids.

METHODS EMPLOYED: Guinea pigs are treated with daily subcutaneous doses of radioactive methyl mercuric chloride (C^{14}), 2 mg/kg of body weight, for a period of seven days. The cochlear potentials are measured and methyl mercury concentrations in the endolymph, perilymph, cerebrospinal fluid and blood are determined with a liquid scintillation counter.

MAJOR FINDINGS: The sound pressure levels in the external ear canal of guinea pigs were measured in a frequency range from 0.25 KHz to 20 KHz by utilizing a probe tube microphone. The intensity functions of cochlear microphonics recorded in the basal and third turn were obtained and statistical analysis was carried out. The method of collection of endolymph was developed in order to minimize contamination of perilymph and blood. A double-barreled capillary was used for collection of the endolymph. One barrel was used to record the endocochlear potential and the other barrel was used to sample the endolymph. The data from methyl mercury-treated animals will be obtained in the next fiscal year.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: It has been reported that methyl mercury has a selective toxicity to the outer hair

cells in the middle turn of the guinea pig cochlea. This project will provide additional knowledge regarding the ototoxic mechanism of methyl mercury.

PROPOSED COURSE: This project will be carried out as outlined.

KEYWORD DESCRIPTORS: Methyl mercury, guinea pigs, endolymph, perilymph, cochlear potentials.

Project No.: Z01 ES 50003-02 EBpB
1. Environmental Biophysics Branch
2.
3. Research Triangle Park, N. C.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

PROJECT TITLE: "Physiological Study of Auditory Fatigue"

PREVIOUS SERIAL NUMBER: NIEHS-EB-008

PRINCIPAL INVESTIGATOR: Teruzo Konishi, M.D., Ph.D.

OTHER INVESTIGATORS: Alexandra Yankwich, M.S., Regina O. Cook

COOPERATING UNIT: None

MAN YEARS

Total:	1.4
Professional:	0.6
Others:	0.8

PROJECT DESCRIPTION

OBJECTIVES: The objective of this project is to explore the location and nature of physiological changes underlying the temporary threshold shift.

METHODS EMPLOYED: The cochlear microphonic (CM), whole-nerve action potential (AP) and endocochlear potential (EP) were recorded in anesthetized guinea pigs. The recovery processes of these cochlear potentials were examined after sound exposure. The effects of exposure tones and test tones on the recovery process were systematically tested.

MAJOR FINDINGS: CM did not change its magnitude during and following the exposure to 8 KHz for a period of two minutes when the exposure level was kept below 100 dB SPL. The exposure level above 100 dB SPL resulted in a progressive decline of CM and hysteresis of EP during the exposure. The input-output curve of CM recorded after sound exposure demonstrated downward shift. A positive summing potential was frequently observed during the recovery period after exposure to intense sound level. AP was more vulnerable to overstimulation than CM. The suppression of AP in response to low level of test tone (40 dB SPL) was greater than to high level of test tones (80 dB SPL). When the exposure level is moderate, AP showed supernormality during the initial phase of the recovery process in response to test tone of low intensity.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The ability of an ambient sound to produce permanent hearing loss over a long period of time can be predicted from the temporary threshold shift (TTS)

based-damage risk criteria). The knowledge obtained by this project is important for an understanding of the physiological process upon which the temporary threshold depends.

PROPOSED COURSE: The recovery process of single-auditory-nerve fiber response is measured in anesthetized guinea pigs or cats. The experimental paradigms described in "method" are used in guinea pigs or cats which are treated with subtoxic doses of kanamycin (200 mg/kg, 5 days) in order to test the possible potentiation effect of the antibiotics on the auditory recovery process after sound exposure.

KEYWORD DESCRIPTORS: Auditory fatigue, guinea pig, cochlear microphonic, action potential, summating potential, endocochlear potential.

Project No.: Z01 ES 50004-02 EBpB
1. Environmental Biophysics Branch
2.
3. Research Triangle Park, N. C.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

PROJECT TITLE: "Effect of Microwave Radiation on Auditory System"

PREVIOUS SERIAL NUMBER: NIEHS-EB-010

PRINCIPAL INVESTIGATOR: Teruzo Konishi, M.D., Ph.D.

OTHER INVESTIGATORS: Donald I. McRee, Ph.D.
Philip E. Hamrick, Ph.D.
Reginald O. Cook
Alexandra Yankwich, M.S.

COOPERATING UNIT: None

MAN YEARS

Total: .20
Professional: .20
Other: .00

PROJECT DESCRIPTION

OBJECTIVES: The objective of this project is to determine whether microwave radiation causes functional or metabolic disturbance in guinea pig's cochlea.

METHODS EMPLOYED: The stimulus-related responses and endocochlear potential are recorded in the guinea pig cochlea. The intracochlear temperature is measured by a thermistor. Animals are exposed to modulated microwaves of frequencies from 1 to 10 GHz. The electromagnetic field intensity ranges from 2 to 10 mW/cm². The stimulus-response relationships are tested. Exposure is for four hours per day for 30 days. The cochlear potentials are measured in the first and fifth week after the exposure to microwave radiation. The histological changes of the temporal bone are studied.

MAJOR FINDINGS: The effort put into this project was the development of a technique to expose unanesthetized guinea pigs to uniform electromagnetic fields. Due to an unexpected breakdown of the microwave exposure system, this project was delayed and there are no major findings to report at this time.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: This project is an attempt to better understand the effect of microwave radiation on the auditory system and to investigate the nature of functional alteration of the auditory end-organ induced by microwave radiation.

PROPOSED COURSE: This project will be carried out as outlined.

KEYWORD DESCRIPTORS: Microwave radiation, guinea pig, cochlear potentials.

Project No.: Z01 ES 50005-02 EBpB
1. Environmental Biophysics Branch
2.
3. Research Triangle Park, N. C.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

PROJECT TITLE: "Studies of Energy Balance and Metabolism in Cochlea Affected by Noise, Ethacrynic Acid and Other Ototoxic Agents"

PREVIOUS SERIAL NUMBER: NIEHS-EB-009

PRINCIPAL INVESTIGATOR: Teruzo Konishi, M.D., Ph.D.

OTHER INVESTIGATORS: Insu Lee, Ph.D., Alexandra Yankwich, M.S., Reginald Cook

COOPERATING UNIT: Environmental Toxicology Branch

MAN YEARS

Total:	.20
Professional:	.20
Other:	.00

PROJECT DESCRIPTION

OBJECTIVES: The objectives of this project are to study changes in energy utilization and production under the influence of ototoxic agents and to reveal the specific mechanism by which these actions occur in the cochlea.

METHODS EMPLOYED: The cochlear microphonic and action potentials of the auditory nerve are recorded and the endocochlear potential is monitored in anesthetized guinea pigs. The permeability of the cochlear partition to Na and K is determined. The results obtained in animals exposed to ototoxic agents are compared with those in controls.

MAJOR FINDINGS: Because of a delay of development of the laboratory set up, and unexpected staffing delay, we were unable to initiate this project. No results are available as yet.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Ototoxicity of the subject agents has been studied but bio-chemical basis underlying the alteration of the cochlear function is not fully understood. The results obtained by this project will permit us to identify and begin to understand the basic mechanism of cochlear pathology and resulting hearing impairment.

PROPOSED COURSE: We plan to assay ATP levels in stria vascularis, if techniques can be developed.

KEYWORD DESCRIPTORS: Noise exposure, ethacrynic acid, ototoxic agents, permeability of cochlear partition, sodium in endolymph, potassium in endolymph.

Project No.: Z01 ES 50006-01 EBpB
1. Environmental Biophysics Branch
2.
3. Research Triangle Park, N.C.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

PROJECT TITLE: "Response of the Inner Ear to Speech When Delivered 1) In a Normal Acoustic Manner 2) When the Same Source Waveforms Including Speech are Delivered via PZT Drivers"

PREVIOUS SERIAL NUMBER: None

PRINCIPAL INVESTIGATOR: Reginald O. Cook

OTHER INVESTIGATORS: Teruzo Konishi, M.D., Ph.D., W.G. Thomas, Ph.D.

COOPERATING UNIT: Speech and Hearing Clinic, University of North Carolina at Chapel Hill

MAN YEARS

Total:	.25
Professional:	.20
Other:	.05

PROJECT DESCRIPTION

OBJECTIVES: The overall objective was to ascertain whether piezoelectric transducers (PZT) or other direct contact transducers could impart complex signals to the cochlea and how these would compare in discrimination tests with identical source waveforms presented in the normal acoustic manner.

METHODS EMPLOYED: The differential electrode technique, very precisely calibrated stereo tape recorders and an extremely sensitive but small size microphone which was placed very close to the tympanic membrane of the animal. Phonetically balanced word (PB) lists and other standardized speech phrases were acoustically delivered to the tympanic membrane of guinea pigs and the resulting cochlear microphonic and output of the external microphone recorded simultaneously on the tape recorder. The signal, (including the phonetically balanced word lists and other speech segments) recorded from the microphone were then used as the input electrical signal to drive the PZT drivers. These drivers were made to directly vibrate the ossicular chain at the umbo thus imparting the speech. The cochlear microphonic generated in response to this speech (the directly generated displacement) was then recorded both for the PZT beam and rod. Next a panel of listeners were asked to discriminate the speech according to a standard test developed for the evaluation of hearing aids, namely PB word recognition. The same panels were also asked to rate the other speech segments according to a scale developed by us. While some data is still being statistically evaluated

no fidelity differences appear to exist.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: With respect to hearing aids, it is common knowledge to otologists, audiologists and users that the common ordinary hearing aid is a very poor fidelity device and the problems with it which are principally imposed by size constraints are such that we are not likely to see a great improvement in the foreseeable future. The reasons for improvements standstill is related to the fact that a very tiny acoustic diaphragm is used to generate sound in the hearing aid. To ask such a tiny diaphragm to perform acceptably over a wide amplitude range and a wide range of frequencies is asking it to buck many of the immutable laws imposed on sound generating devices. These laws say essentially that for efficient production of sound particularly in the low frequencies, a fairly large vibrating object is necessary. Unfortunately, the low frequency area is the only place that many people have any residual hearing left. In addition to this problem, one has a problem of feedback, squeal problems inherent when microphone and speaker are placed very close, plus other problems too numerous to mention. It has long been thought by otologists that implanted devices might be capable of imparting speech directly to the inner ear by driving the ossicular chain. It was postulated that this could be done by a tiny transducer because the difficult mechanical-to-acoustic step is bypassed. But no one has heretofore extensively investigated the fidelity which might be possible by using such devices. This project has successfully demonstrated that insofar as fidelity is concerned, these devices are capable of the same order of fidelity as possible by the normal acoustic method itself and that such problems as now stand between the successful implantation of these devices are essentially related to solving the implantation problems themselves.

PROPOSED COURSE: This device will be utilized in certain situations requiring close monitoring of the waveform imparted to the inner ear and in situations involving the need for excess overstimulation which is difficult to achieve acoustically. Acute implantations will be carried out in animals.

A paper on this work is in preparation and will be given at the fall meeting of the Acoustical Society of America.

KEYWORD DESCRIPTORS: Hearing aids, PZT, cochlea, response.

Project No.: Z01 ES 50007-03 EBPB
1. Environmental Biophysics Branch
2.
3. Research Triangle Park, N.C.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

PROJECT TITLE: "Simultaneous Measurement of Ossicular Chain Movement, Free Field Sound Pressure Level at the Tympanic Membrane and Cochlear Electrical Potentials in Response to 1) Sound and 2) Piezoelectric and other Direct Contact Transducers"

PREVIOUS SERIAL NUMBER: NIEHS-EB-005

PRINCIPAL INVESTIGATOR: Reginald O. Cook

OTHER INVESTIGATOR: Teruzo Konishi, M.D., Ph.D.

COOPERATING UNIT: None

MAN YEARS

Total:	.50
Professional:	.35
Other:	.15

PROJECT DESCRIPTION

OBJECTIVE: Overall objective is to ascertain whether a PZT device or direct contact (but not bonded) transducer will impart the same signal to the cochlea as an acoustic pure tone wave when both are set to create equal maximum displacement of the ossicular chain, or otherwise characterized, would each of the two different methods require the ossicular chain to undergo the same displacement and waveform pattern for equal inner ear output (cochlear microphonic) for pure tones.

METHODS EMPLOYED: Using techniques heretofore developed in projects NIEHS-RSB-009 and NIEHS-RSB-024 in combination with the placement of differential cochlear microphonic electrodes, we compared the input and output waveforms. Displacements were measured with a non-contact fiber optic technique wherein light is imparted to one end (and one bundle) of a bifurcated fiber bundle. The light reflected from the vibrating target at the other end, was measured by a photosensor at which had been terminated the other bundle of the bifurcated optic fiber complex. Light pipes, i.e., fiber optics bundles are capable of being used as optic levers since light emerges at the same angle as which it enters and as a vibrating target moves into and away from the fiber optic bundle tip, more or less light is reflected back into the receiving fibers, which in turn are sensed by the photosensor. Sound pressure levels were measured with probe tube microphones terminated at the point of entry to the tympanic

membrane. Sound pressure was generated free field and sufficient sound to hold constant cochlear microphonic at the tympanic membrane was provided by means of a feed back regulating amplifier which controls the amplifier output power to the loud speaker.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: We have demonstrated capability to measure the three parameters necessary for characterizing the response of the ear to sound, namely the sound pressure level occurring at the ear drum, the displacement at the umbo which indicates the response of the middle ear to the sound pressure level, and the cochlear microphonic which is a very good indicator at moderate sound levels and intensities of the response of the inner ear to the velocity and the waveform present at the stapes. It is difficult to over estimate the significance of having all three parameters measured simultaneously and no such simultaneous data has been previously reported. It is pushing the state of the art to measure displacement sensitivities of the levels necessary, i.e., 70-80 angstroms, within the range of linearity of the cochlear microphonic. Previous data has been limited to sound pressure versus cochlear microphonic and in a few cases of sound pressure versus displacement. Other methods of measuring displacement at the sensitivity necessary have required instruments too complex to easily set up for simultaneous cochlear microphonic and sound pressure measurements.

PROPOSED COURSE: Techniques developed in this project will be routinely utilized and extended in our noise effects/auditory physiology investigations.

A paper on earlier phases of this work was given at the annual meeting of the Acoustical Society of America and several papers on the later work are in preparation.

KEYWORD DESCRIPTORS: Tympanic membrane, cochlear potentials, piezoelectric transducers.

Project No.: Z01 ES 50008-02 EBpB
1. Environmental Biophysics Branch
2.
3. Research Triangle Park, N.C.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

PROJECT TITLE: "Development of a Double Beam Laser Microscope for Recording
Ossicular Chain and Basilar Membrane Displacement and Waveform"

PREVIOUS SERIAL NUMBER: NIEHS-EB-006

PRINCIPAL INVESTIGATOR: Reginald O. Cook

OTHER INVESTIGATORS: Geza Jako, M.D. and Teruzo Konishi, M.D., Ph.D.

COOPERATING UNIT: Boston University School of Medicine

MAN YEARS

Total:	.50
Professional:	.35
Other:	.15

PROJECT DESCRIPTION

OBJECTIVES: Objective was to develop a calibrated electro-optic microscope utilizing twin laser beams so that phase and amplitude differences for two closely adjacent points on the vibrating basilar membrane can be simultaneously measured. Amplitude sensitivity sought is on the order of 1 angstrom unit.

METHODS EMPLOYED: Using a metallurgical microscope and a high power, long working distance lens coupled with twin laser beams brought "together" by use of a rhomboidal prism mechanism and a state of the art electro-optic photo sensor, we achieved ability to measure displacement of 1 angstrom off PZT drivers. A paper detailing the method was given at the May 1974 meeting of the Acoustical Society of America.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE:

Quantitative characterization of the basilar membrane response to pressure differences set up in the cochlea by the velocity and the waveform at the stapes have long been sought by people working in auditory physiology/noise effects. Since the information which must be generated from a knowledge of the displacement of waveform in the basilar membrane is necessary to fill in great gaps in auditory theory regarding what information is being processed hydrodynamically and mechanically versus what must be elicited higher up in the nervous system. The project was shelved for most of the period reported on in favor of the fiber optic technique mainly because of size differences in the respective sensors. While the lazer microscope technique is capable of greater sensitivity; the

utility and small size of the fiber optic technique made it more expeditious for us to use it for most of the experiments involved in NIEHS-EB-005.

PROPOSED COURSE: The work will be continued as time permits, however, because it appears that this will be a good way to measure the response of the apical end of the cochlea where it is possible to directly impart light on the basilar membrane. In the basal end of the membrane, a complex light path utilizing tiny mirrors is necessary and presents formidable problems.

KEYWORD DESCRIPTORS: Hearing, ossicular chain, displacement measurement, laser, microscope.

Project No.: Z01 ES 50009-02 EBpB
1. Environmental Biophysics Branch
2.
3. Research Triangle Park, N. C.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

PROJECT TITLE: "Noise and Cell Mediated Immunity"

PREVIOUS SERIAL NUMBER: NIEHS-EB-013

PRINCIPAL INVESTIGATOR: Sherman Fox, M.D.

OTHER INVESTIGATOR: Reginald O. Cook

COOPERATING UNIT: None

MAN YEARS

Total:	1.3
Professional:	.9
Other:	.4

PROJECT DESCRIPTION

OBJECTIVES: In the experimental animal, acute noise exposure has been shown to markedly affect various hormonal secretions, among them adrenal steroids and thyroid hormones. Immunologic function has been shown to be affected by changes in levels of these hormones. This serves as a rationale for studying possible effects of noise on immune system function. Work in the early 1960's showed that prolonged high intensity noise increased the susceptibility of mice to viral infections. This was later shown to correlate with impairment of interferon production. Subsequent work by Russian scientists has shown various immunological deficiencies, both in experimental animals and in human workers, after chronic noise exposure. The objective of this project is to attempt to replicate the Russian findings concerning noise and cell-mediated immunity, and to further delineate the locus of action.

METHODS EMPLOYED: Cell-mediated immunity will be measured in male rats in vivo by de novo sensitization to PPD. Sensitization will be attempted during various patterns of constant and intermittent noise. The noise level will be 100 dBA, and the intermittent noise will consist of two, five minute periods per hour, 15 consecutive hours per day.

Cell mediated immunity will also be measured in vitro by lymphocyte stimulation by phytohemagglutinin. This will be measured by a micromethod, requiring only 0.5 ml of peripheral blood. As a result, we will collect sequential data on a single animal during lengthy noise exposure. Again, both constant and intermittent noise will be looked at.

MAJOR FINDINGS: Work with various patterns of noise exposure has indicated that sensitization does not require "noise-sensitive" precursors. Constant noise exposure between sensitization and challenging dose, however, has led to a statistically significant decrease in the amount of reaction to PPD. Early data shows a marked diminution of lymphocyte stimulation following noise exposure.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: This is an attempt to better understand the influence of environmental stresses on the host-parasite relationship. In addition, it is an attempt to study one parameter of noise, intermittency, that more closely approximates its environmental occurrence than previous experimental models.

PROPOSED COURSE: We are now attempting to replicate and collect more data in the lymphocyte stimulation work.

KEYWORD DESCRIPTORS: Noise and cell-mediated immunity; intermittency of noise.

Project No.: Z01 ES 50010-02 EBpB
1. Environmental Biophysics Branch
2.
3. Research Triangle Park, N. C.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

PROJECT TITLE: "Multielement Analysis Using X-ray Emission Spectrometry"

PREVIOUS SERIAL NUMBER: NIEHS-EB-015

PRINCIPAL INVESTIGATORS: P. J. Walsh, Ph.D. and P. Hamrick, Ph.D.

OTHER INVESTIGATORS: None

COOPERATING UNITS: None

MAN YEARS

Total:	1.1
Professional:	.9
Other:	.2

PROJECT DESCRIPTION

OBJECTIVES: This project was initiated to develop quantitative techniques for the determination of trace amounts of elements, singly and in combination, in biological and environmental samples.

METHODS EMPLOYED: X-ray emission spectrometry has the advantage of speed of analysis because of its ability to analyze many elements simultaneously and its high detection efficiency. In recent years, the combination of semiconductor detectors with field-effect-transistor input preamplifiers and the optimization of that combination has yielded the resolution necessary to allow separation of the characteristic X-rays for all elements above atomic number 12.

MAJOR FINDINGS: A system incorporating a lithium-drifted germanium detector provides a minimum detectability of 2 μgm in 100 seconds. A new system promising better sensitivity is ready for testing.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Aside from its value as a conventional analytical tool, X-ray emission spectrometry offers the possibility of in vivo studies in animals and man ranging from the study of the distribution of iodine in thyroid glands to measurement of deposition of aerosols in the respiratory tract. A variety of studies normally carried out by the use of radioactive tracers appear within the capabilities of X-ray emission spectrometry.

PROPOSED COURSE: This project has been terminated.

KEYWORD DESCRIPTORS: Multielement analysis, X-ray emission spectrometry.

Project No.: Z01 ES 50011-03 EBpB
1. Environmental Biophysics Branch
2.
3. Research Triangle Park, N.C.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

PROJECT TITLE: "Theoretical Basis of Dose-Response Relationships"

PREVIOUS SERIAL NUMBER: NIEHS-EB-014

PRINCIPAL INVESTIGATOR: P.J. Walsh, Ph.D.

OTHER INVESTIGATORS: P.E. Hamrick, Ph.D.

COOPERATING UNITS: None

MAN YEARS

Total:	2.5
Professional:	1.5
Other:	1.0

PROJECT DESCRIPTION

OBJECTIVES: The objective of this project is to develop a mathematical-theoretical model of the effects of ionizing radiation at the cellular level.

METHODS EMPLOYED: Experimental and theoretical approaches have been developed. Experimental systems incorporating spectrometry systems have been designed to facilitate the delivery of measured doses of alpha, beta or gamma radiation to cell cultures. A theoretical treatment, built upon data from the literature, can be used to focus the experimental work and to predict the nature of dose-response relationships under low-dose, low-dose rate conditions.

MAJOR FINDINGS: The theoretical model agrees with experimental cell survival curves available in the literature. This model treats the relationships among such parameters as dose, dose rate, cell turnover time and altered cell division and repair. Several other models arise as special cases of the model. The parametric values obtained from cell survival data may be used to describe cell alteration as a function of dose. Further experimental work is needed to specifically test the model under low-dose, low-dose rate conditions.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The development of theoretical models is necessary to improve understanding of the connections between damage to cells and subsequent biological effects.

PROPOSED COURSE: Experimental work on model development may be begun by contract. No in-house experimental work is underway at present.

KEYWORD DESCRIPTORS: theoretical model, ionizing radiation, cellular level

PUBLICATIONS

Walsh, P.J.: On the application of the uncertainty principle to stopping power theories. Health Physics 19:574, 1970.

Walsh, P.J.: Stopping power and range of alpha particles. Health Physics 19:312-316, 1970.

Walsh, P.J.: Possible effects of dose rate and turnover time of cells on dose response relationships: Implications for radiation protecting guidance. International Journal of Environmental Studies 2:189-193, 1971.

Walsh, P.J.: Energy loss of alpha particles in tissue equivalent plastic. Health Physics 23:701-704, 1972.

Walsh, P.J.: Possible effects of division and repair of altered cells on dose response relationship in the context of carcinogenesis. International Journal of Environmental Studies 5:285-288, 1974.

Walsh, P.J.: On the relationship between cell survival and cell alteration after exposure to ionizing radiation. (In press)

Project No.: Z01 ES 50012-03 EBpB
1. Environmental Biophysics Branch
2.
3. Research Triangle Park, N.C.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

PROJECT TITLE: "Measurement of Energy Absorption from Microwave Fields"

PREVIOUS SERIAL NUMBER: NIEHS-EB-016

PRINCIPAL INVESTIGATOR: P. J. Walsh, Ph.D.

OTHER INVESTIGATORS: Daryl Mercer, Graduate Student, UNC
Ravi Matthew, Graduate Student, UNC

COOPERATING UNITS: Department of Environmental Sciences & Engineering, UNC

MAN YEARS

Total:	1.2
Professional:	1.2
Other:	0.0

. PROJECT DESCRIPTION

OBJECTIVE: Measurement of the absorption of microwave energy on the basis of fundamental physical laws.

METHODS EMPLOYED: Microwaves are absorbed in a spherical shell of dielectric material enclosing a dry gas or a spherical shell of dry gas enclosing the dielectric. Temperature changes of the dielectric are transmitted to the gas which expands in accord with the ideal gas law over the range of pressures and temperatures encountered.

MAJOR FINDINGS: A double-walled spherical glass bulb in current use with saline dielectric and dry air gas exhibits sensitivities of $100\text{--}200\mu\text{ W/cm}^2$, a factor of 100 below current U.S. exposure limits. An investigation of the utility of the system for determining the microwave absorption properties of various material is nearing completion.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Measurement of dose or absorbed energy is generally more desirable than measurement of field intensity. A simple device which allows measurement of absorbed energy as well as exhibits adherence to basic physical laws has been a primary goal in radiation dosimetry.

PROPOSED COURSE: This project will be completed by August 1975.

KEYWORD DESCRIPTORS: Microwave energy absorption

Project No.: Z01 ES 50013-05 EBpB
1. Environmental Biophysics Branch
2.
3. Research Triangle Park, N. C.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

PROJECT TITLE: "Variable Frequency Microwave Exposure System"

PREVIOUS SERIAL NUMBER: NIEHS-EB-018

PRINCIPAL INVESTIGATOR: D. I. McRee, Ph.D.

OTHER INVESTIGATOR: None

COOPERATING UNITS: None

MAN YEARS

Total:	.40
Professional:	.20
Other:	.20

PROJECT DESCRIPTION

OBJECTIVES: The objective of this project is to design, build and calibrate a variable frequency exposure system for biological material.

METHODS EMPLOYED: Specifications were written for the desired system. A field uniformity at the specimen of 90 percent over a 4-inch diameter circle, a continuous frequency variation in the range of 1 to 10 GHz and a power level range at the specimen of 0.010 to 10 mW/cm² were specified.

MAJOR FINDINGS: Many of the biological effects of microwave radiation are frequency dependent. In terms of whole body irradiation, a frequency of 150-1,200 MHz affects the internal organs, whereas, a frequency of 10,000 MHz and above affects primarily the skin. Components of cells such as RNA, DNA and protein were found to have maximum absorption at different frequencies. Therefore, it is necessary to have the capability of varying frequency when investigating microwave effects on biological systems. The frequency range of 1 to 10 GHz was chosen because it is within this range of frequencies to which the general population is exposed.

The system has been assembled and is in operation. Biological specimens are presently being exposed using this variable frequency system.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Many biological effects of microwave radiation appear to be frequency specific. The variable frequency exposure system will provide the researchers of the

Institute with the capability of studying the effects of different frequencies and modulated signals on biological systems.

PROPOSED COURSE: This specific project has been completed. Many of the components used in this system can also be used in the assembly of other exposure techniques and in the system to determine the electrical properties of biological material.

KEY WORD DESCRIPTORS: Variable frequency microwave exposure system, biological material, 1-10 GHz, 10 mW/cm².

PUBLICATION

McRee, D.I., Walsh, P.J., and Mathews, R.: Variable frequency exposure system for small biological specimens. (In Press) Review of Scientific Instruments.

Project No.:Z01 ES 50014-06 EBpB

1. Environmental Biophysics Branch
- 2.
3. Research Triangle Park, N.C.

PHS-NIH

Individual Project Report

July 1, 1974 through June 30, 1975

PROJECT TITLE: "Microwave Exposure System and Microwave Dosimetry"

PREVIOUS SERIAL NUMBER: NIEHS-EB-017

PRINCIPAL INVESTIGATOR: D.I. McRee, Ph.D.

OTHER INVESTIGATOR: P.J. Walsh, Ph.D.

COOPERATING UNIT: None

MAN YEARS

Total:	.40
Professional:	.20
Other:	.20

PROJECT DESCRIPTION

OBJECTIVES: The objectives of this project are to develop a microwave exposure system for biological material which has a well-defined, uniform field characteristic; and to develop dosimetric relationships for energy absorption.

METHODS EMPLOYED: A thorough study of the research requirements on the biological effects of microwave was completed. Based on these studies, a set of specifications was written for a microwave exposure system which would generate a uniform, low-level microwave field in a small laboratory space. At present, it is difficult to accurately measure low level microwave fields and to determine the mechanisms by which the fields interact with matter. It is necessary, therefore, to conduct dosimetric studies so as to develop analytical interaction models. Microcalorimetry, thermistor and thermocouple detectors, chemical reactions, liquid crystals and infrared microscopes are some of the techniques which will be investigated. Other instruments for measuring incident and absorbed energy will be evaluated as they become available.

MAJOR FINDINGS: A microwave exposure system with a frequency of 2450 MHz has been designed, built and the field calibrated. The microwave field at the specimen location was determined to be uniform within the design specifications with variation in power density as follows:

6 inch diameter area	- $\pm 5\%$
9 inch diameter area	- $\pm 10\%$
12 inch diameter area	- $\pm 15\%$

The system provides a power density range of 0.01 mW/cm^2 to 200 mW/cm^2 .

Thermistors and thermocouples have been evaluated for interaction with the microwave field. The thermistor and thermocouples were placed in the field with only air as the surrounding medium. The thermistor was not affected whether the leads to the thermistor bead were or were not shielded and/or grounded. The thermocouple was very sensitive to microwave interference if the leads were not grounded. Even when grounded some instability was noted. In future absorption and biological effects research, the thermistor will be used to monitor temperature.

An infrared microscope which can focus to a spot size of 0.25 cm at 52 cm from the aperture was obtained and evaluated. The surface temperature of objects being exposed to microwaves can be measured to $\pm 0.5^\circ\text{C}$ using this instrument. Liquid crystals with a range of $33\text{--}37^\circ\text{C}$ were tested for their utility and accuracy. In this temperature range it was found that the surface temperature could be obtained to an accuracy of $\pm 1^\circ\text{C}$.

Mathematical model techniques for calculating time-temperature profiles for biological specimens which have been exposed to microwave radiation were developed. The mathematical models can be used to calculate energy absorption and temperature increases of a body when exposed to different levels of microwave radiation.

The exposure area of specimen location has been placed in a microwave absorber-lined environmental chamber. This achievement provides the added capability of adjusting the environmental temperature and humidity at the specimen and greatly increases the versatility of the system.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The well defined microwave field will allow the Institute to perform accurate quantitative studies of the effects of microwave radiation in biological systems at a frequency of 2450 MHz . Using the thermistor to measure the temperature in the controls using ordinary heating techniques. This procedure will enable us to separate thermal and non-thermal effects.

PROPOSED COURSE: A 2450 MHz pulsed microwave source has been ordered for the 2450 MHz system. This source will allow the peak power of study of the biological effects of both CW and pulsed radiation. The pressure transducer-capillary detector which has been fabricated will be tested as to its accuracy, sensitivity, and response time. Implantable electric and magnetic field probes will be investigated as they become available. A prototype fiber optic-liquid crystal probe developed by the University of Utah and Office of Naval Research will be evaluated in our laboratory. It is reported that this probe provides an accurate measure of temperature and does not perturb the field at the measurement location.

KEYWORD DESCRIPTORS: Microwave exposure system, microwave dosimetry, microcalorimetry, thermistor, infrared microscope.

PUBLICATIONS

McRee, D.I.: Thresholds for lenticular damage in the rabbit eye due to single exposure to CW microwave radiation: An analysis of the experimental information at a frequency of 2.45 GHz. Health Physics, Vol. 21, Dec. 1971.

McRee, D.I. and Walsh, P.J.: Microwave exposure system for biological specimens. The Review of Scientific Instruments, Vol. 42, No. 12, Dec. 1971.

McRee, D.I. and Pendergrass, F.T.: Interaction of a 2450 MHz microwave field with thermocouples and thermistors. Health Physics, Vol. 25, August, 1973.

McRee, D.I.: Determination of the absorption of microwave radiation by a biological specimen in a 2450 MHz microwave field. Health Physics, Vol. 26, May, 1974.

McRee, D.I.: Determination of energy absorption of microwave radiation using the cooling curve technique. Journal of Microwave Power, Vol. 9, No. 3, September, 1974.

Project No.: Z01 ES 50015-02 EBpB
1. Environmental Biophysics Branch
2.
3. Research Triangle Park, N.C.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

PROJECT TITLE: "Effects of Microwave Radiation on the Neural Response"

PREVIOUS SERIAL NUMBER: NIEHS-EB-022

PRINCIPAL INVESTIGATOR: Donald I. McRee, Ph.D.

OTHER INVESTIGATOR: George Somjen, M.D., Duke University

COOPERATING UNIT: Duke University, Department of Electrical Engineering,
Department of Physiology

MAN YEARS

Total:	.25
Professional:	.15
Other:	.10

PROJECT DESCRIPTION

OBJECTIVE: The objective of this project is to determine the effects of microwave radiation on the functioning of the central nervous system when exposed to CW and modulated microwave radiation in the frequency range of 1-10 GHz.

METHODS EMPLOYED: Isolated neurons such as the abdominal ganglion of the Aplysia, the sciatic nerve of the frog, and the saphenous nerve of cats will be exposed to CW and modulated microwave radiation in the power density range of 1-10 mW/cm². The effects of the radiation on cell membrane potentials, action potential and conduction velocity will be investigated.

More complex systems such as the exposed spinal cord of decerebrated cats will be exposed. These test systems will be used to investigate the effects of radiation on synaptic neurotransmitters.

MAJOR FINDINGS: A review of the literature indicates that the brain and central nervous system are sensitive to microwave radiation. Many of the behavioral effects reported on animals and man result from CNS interactions. In most of the studies no explanation of the mechanism by which the microwave interacts with nerve cells is specified.

The spinal cords of 14 cats were exposed directly to microwaves by the removal of muscle and column enclosing the spinal cord. The frequency of the CW radiation was 2450 MHz and the incident power density was 10 mW/cm². By

stimulating the sciatic nerve in the leg of the cat and observing the action potential in the ventral nerve (sensory root) the effects of the radiation on reflex response and synaptic function could be determined. The data showed that a small but statistically significant enhancement of reflex transmission was caused by the radiation. The average increase in response was of the order of 10 percent.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The potential health effects of microwave radiation in the environment is of prime interest to NIEHS. The neurological behavior effects reported in the literature illustrate the need for significant effort in this area. At present the accepted safe level of exposure in the U.S. is 1000 times greater than the standard in the U.S.S.R. Their standard is based on neurological and behavioral response to microwave radiation. This research on the effects of microwave on CNS is directed toward the mission of the Institute to determine the health effects of physical factors in the environment.

PROPOSED COURSE: The spinal cord of cats will be exposed to 2450 MHz CW radiation at 20 mW/cm^2 to determine if the effect is increased with increased exposure level. Other neural specimens will be exposed to CW, pulsed, and modulated microwave fields. The effect on action potentials, firing potentials, conduction velocity and signal transmission will be measured.

KEYWORD DESCRIPTORS: Microwave radiation, neural response, isolated neurons, spinal cord, cats.

Project No.: Z01 ES 50016-04 EBpB

1. Environmental Biophysics Branch
- 2.
3. Research Triangle Park, N.C.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

PROJECT TITLE: "Effects of 2450 MHz Microwave Radiation on Biological Materials at Cellular Level"

PREVIOUS SERIAL NUMBER: NIEHS-EB-020

PRINCIPAL INVESTIGATOR: P.E. Hamrick, Ph.D.

OTHER INVESTIGATORS: J. Zinkl, D.V.M, EBCB, Sherman Fox, M.D.

COOPERATING UNIT: EBCB

MAN YEARS:

Total:	1.1
Professional:	0.5
Other:	0.6

PROJECT DESCRIPTION

OBJECTIVES: The objectives of this project are to determine how 2450 MHz microwave radiation interacts with biological material at the cellular and macromolecular level, to observe any effects of this interaction and to relate the amount of microwave energy absorbed to the effects.

METHODS EMPLOYED: Various cellular and macromolecular systems are exposed to microwave radiation at carefully controlled temperatures and exposure levels. Much of the work has been at 37°C. Some biological systems employed in the study are: bacteria and bacteriophage, solutions of DNA, 2-4 cell stage embryos from mice, rabbit erythrocytes and human and mouse lymphocytes.

MAJOR FINDINGS: Studies have been made which suggest that "non-thermal" microwave fields (fields which do not significantly increase the temperature of the specimen) at various frequencies interact with intracellular components to alter metabolic and/or genetic processes. Interpretation of these effects is difficult because mechanisms are not specified and in many cases the exposure parameters are not known quantitatively. Many of the effects appear to be very frequency dependent. For example, the growth rate of Escherichia coli has been shown by others to be enhanced or retarded depending on the frequency in the range of 65-75 GHz. In the past, when Escherichia coli and Pseudomonas aeruginosa were exposed in our lab to 2450 MHz microwaves at 60 mW/cm² and held at 37°C, no effects on growth rate were observed. Under similar conditions no effect was observed on the lysis time of bacteriophage

T-2, on the denaturation curves of DNA or which was teratogenic when mouse embryos were exposed. These studies have indicated that if there are "non-thermal" effects at 2450 MHz then these effects are relatively small in the systems we have investigated. Polish and Russian scientists report effects on membrane transport in rabbit erythrocytes and stimulation of isolated lymphocytes to undergo transformation to a blastocysts-like cell. During the past year, the rabbit erythrocyte work has been repeated as nearly like the Polish work as possible. We were able to very closely duplicate the experiments since we had the opportunity to discuss the project with Polish scientists familiar with the work. Some of the parameters tested (such as K^+ efflux) were found to be highly temperature sensitive but no effects on membrane permeability were detected that were not due to temperature changes. Other studies have been started with lymphocytes.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Before an accurate evaluation of the biological effects of 2450 MHz microwaves can be made, it is necessary to carefully control the temperature of the specimen and be able to reproduce the exposure conditions. We have the capability to do this at NIEHS and can relate observed effects of microwaves to purely thermal effects.

PROPOSED COURSE: We plan to continue our study with lymphocytes since stimulation would suggest immunological effects with possible profound effects on animal systems. We will also study other systems in which sensitivity to microwaves has been reported or which are desirable as test systems for microwaves.

KEYWORD DESCRIPTORS: Bacteria, bacteriophage, DNA, embryos, erythrocytes, lymphocytes, 2450 MHz, microwave.

PUBLICATIONS

P.E. Hamrick, Thermal Denaturation of DNA Exposed to 2450 MHz Microwave Radiation, Radiation Research, 56: 400-404, 1973.

P.E. Hamrick and B.T. Butler, Exposure of Bacteria to 2450 MHz Microwave Radiation, Journal of Microwave Power, 8: 227-233, 1973.

P.E. Hamrick and J.G. Zinkl, Exposure of Rabbit Erythrocytes to Microwave Radiation, Radiation Research, 62: 164-168, 1975.

Project No.: Z01 ES 50017-02 EBpB
1. Environmental Biophysics Branch
2.
3. Research Triangle Park, N.C.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

PROJECT TITLE: "Effects of 2450 MHz CW Microwave Radiation on the Embryonic Development of Japanese Quail"

PREVIOUS SERIAL NUMBER: NIEHS-EB-019

PRINCIPAL INVESTIGATORS: P.E. Hamrick, PH.D. and D.I. McRee, Ph.D.

OTHER INVESTIGATORS: J. Zinkl, D.V.M., EBCB, P. Thaxton, Ph.D.,
C. Parkhurst, Ph.D., NCSU

COOPERATING UNITS: EBCB and Poultry Science Department of NCSU (consultants)

MAN YEARS

Total:	1.5
Professional:	0.8
Other:	0.7

PROJECT DESCRIPTION

OBJECTIVES: The objectives of this project are to determine the effects of 2450 MHz CW microwave radiation on the embryological development of Japanese quail.

METHODS EMPLOYED: Fertilized Japanese quail eggs are exposed to 2450 MHz CW microwave radiation at various times and durations during the incubation period. Temperature and humidity conditions are carefully controlled and may be varied to suit the experimental protocol. The newly hatched quail are examined for changes in percent hatched, gross deformities and several blood parameters. The blood parameters studied were hematocrit, hemoglobin, erythrocyte and leukocytes number and differential leukocyte percentages.

MAJOR FINDINGS: Reported data from the literature on the exposure of fertilized chicken eggs to microwave radiation has shown gross deformities. Due to the power density levels and the temperature of the eggs during exposure, there is controversy over whether the effects were thermal or non-thermal. In the majority of our experiments, we have not allowed the temperature of any part of the exposed egg to be greater than 37°C and the exposures have been during the first 5 days of incubation. Experiments have been done in which exposures have been made for 4 hours during the first 5 days or for 24 hours on the second day of incubation. Monitoring was done with thermistors and with an infrared microscope. Eggs were chosen near the same size to obtain

essentially uniform heating. Even with careful selection of the eggs, some of the eggs had a temperature of 3 to 4°C below that of a high at 37°C. No effects on hatching or gross deformities were observed. A significantly lower value for hemoglobin was seen for exposure on the second and third day of incubation. Although the average value was significantly lower, there is a large variability in the blood parameters of young quail and the observed values are well within the range of normal variation. Recently, we have begun exposure of the quail eggs for longer periods of time, up to 14 days of continuous exposure under conditions of low intensity (5-10 mW/cm²). In some of the exposures, the temperature is allowed to increase slightly (1-2°C above optimum incubation temperature) to see if there are synergistic as well as purely thermal effects of microwave heating.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The evaluation of whether microwave radiation affects differentiating cell systems is a basic question which must be answered before microwave radiation exposure can be considered safe. This project will assist the Institute in its mission of determining the health effects of physical factors in the environment.

PROPOSED COURSE: Exposure of quail at 2450 MHz CW microwave radiation will be continued under various exposure and environmental conditions.

KEYWORD DESCRIPTORS: 2450 MHz CW microwave radiation, embryonic development, Japanese quail, humidity, temperature, hematocrit, hemoglobin, erythrocyte leukocytes, differential leukocyte.

PUBLICATIONS

D.I. McRee, P. Hamrick, J.Zinkl, P. Thaxton and C. Parkhurst, Some Effects of the Japanese Quail Embryo to 2.45 GHz Microwave Radiation, presented at the New York Academy of Sciences Meeting in New York, February, 1974 and to be published in the annals of the Society.

P.E. Hamrick, J.G. Zinkl, D.I. McRee, P. Thaxton and C.R. Parkhurst, Leucopenia in Neonatal Japanese Quail, accepted for publication in Poultry Science.

ENVIRONMENTAL MUTAGENESIS BRANCH

ENVIRONMENTAL MUTAGENESIS BRANCH
Summary Statement

The primary objective of the Institute's program in mutagenesis is focused on evaluation and definition of risks to both human somatic and germinal tissue resulting from exposure to genetically active environmental chemicals. The Environmental Mutagenesis Branch was organized late in FY72. Because of the worldwide shortage of resources for the rapid development of research in various problem areas associated with environmental mutagenesis, staff members have used a variety of approaches to accomplish its objective. These include:

1. Development of an intramural research program divided into five main areas. Work on particular projects has been carried out by different mechanisms:
 - a. in-house research by staff scientists
 - b. support of work at other institutions in the collaborative research program with staff scientists as project officers
 - c. developing an announcement in collaboration with the extramural program office to solicit grant proposals in special emphasis areas of environmental mutagenesis
2. Development of a coordinated national program in environmental mutagenesis. A variety of mechanisms have been initiated including:
 - a. formation of a DHEW Interagency Panel on Environmental Mutagenesis (IPEM)
 - b. developing workshops and conferences in special emphasis areas
 - c. developing collaborative programs with other institutes within NIH and with other agencies
 - d. developing programs within the Environmental Mutagen Society in which staff members serve as officers, councillors or members
 - e. developing collaborative studies on genetically active environmental chemicals to rapidly develop a data base to assist in evaluating the risk of exposure to man
 - f. developing program reviews at other national laboratories with major programs in environmental mutagenesis.
3. Development of better communication and coordination at the International level. Staff members are associated with several international programs including:
 - a. US-Japan Cooperative Medical Science Program

- b. US-USSR Environmental Protection Agreement
 - c. US-USSR Environmental Health Agreement
 - d. US-Germany Life Sciences Program
 - e. development of international workshops and conferences
 - f. collaboration with IARC (Lyon) on the mutagenicity of chemical carcinogens
4. Development of better mechanisms for information exchange. These include:
 - a. support of the Environmental Mutagen Information Center (EMIC)
 - b. serving as editor, associate editor or members of the editorial board on journals which publish research in the area of environmental mutagenesis
 5. Development of better mechanisms for training young scientists for research careers in environmental mutagenesis at:
 - a. the predoctoral level
 - b. the postdoctoral level

INTRAMURAL RESEARCH PROGRAM

Staffing - Recruiting efforts have continued to develop a strong intramural research program and to make it possible to carry out a strong collaborative research program as well as the other activities listed above. Dr. Larry Valcovic has been recruited to serve as Acting Assistant Branch Chief, Dr. Georges Mohn (who was formerly the Director of the Central Laboratory for Mutagenicity Testing in Freiburg, Germany) is now Head of the Microbial and Plant Genetics Section and Dr. Michael Lieberman (who was formerly working at the National Cancer Institute as a Staff Fellow) is now Head of the Somatic Cell Genetics Section

Intramural Research Program - The intramural staff has been organized into five sections. The major research accomplishments in each section are as follows:

The Microbial and Plant Genetics Section has made considerable progress in the development and improvement of simple and comprehensive tests for gene mutations and other types of genetic damage in microorganisms. Dr. Tong-man Ong has developed a spot test system for detecting reverse mutations at the ad-3 locus of Neurospora crassa. Various tester strains have been selected that exhibit sensitivity to various types of chemical mutagens. This rapid assay will complement the ad-3 forward mutation system earlier developed by Dr. de Serres which has been used by Drs. Ong and de Serres to test the

mutagenicity of several antischistosomal agents, chemical carcinogens and a wide variety of environmental chemicals. These studies are designed primarily as structure-function analyses to determine the correlation between types of genetic damage induced by compounds of similar structure.

Dr. de Serres is also continuing studies on repair-deficient strains of *Neurospora*. There is similarity in the spontaneous mutation rate at the ad-3 locus with both the wild-type and the repair-deficient strains; however, with x-irradiation, the repair-deficient strain required a 5- to 8-fold reduction in dose to give the same induced frequency as the wild-type *Neurospora*. In additional studies with repair-deficient strains, Dr. Ong has shown a variety of sensitivities of various strains to the chemical carcinogen MNNG. Two of the strains, upr-1 and uvs-2, are two and five times more sensitive to mutation induction than wild type.

In another line of work, Dr. Georges Mohn has developed a strain of *E. coli* which measures mutations at several loci. The mutational types detected include point mutations as well as genetic events involving gene deletions of gross DNA regions. Using hycanthone and its analogs studies are in progress to determine the mutagenic sensitivity of this strain. Dr. Mohn is also working to improve the host-mediated assay system. His approach will be to introduce the bacterial tester strain directly into the mammalian blood stream prior to treatment. After treatment and a short incubation time, the cells will be harvested from the liver. This protocol would allow the microbial indicator to remain in the blood stream for a longer period of time and hence mutagenic activities of metabolites of environmental chemicals more clearly detected.

Studies with various microbial systems have further strengthened the correlation between carcinogenic and mutagenic activity. The ad-3 data indicate that the carcinogens tested produce mutations primarily by base-pair substitutions. The utility of microbial mutagenesis assays as a prescreen for chemical carcinogens appears to be very promising.

During the past year, Dr. Michael Lieberman joined the staff as Head, Somatic Cell Genetics Section, and has focused attention on the interaction of mutagens and carcinogens with DNA and synthetic polynucleotides. In one study on the binding of heavy metals it is indicated that different cations may exhibit selectivity in binding to DNA. Additionally, studies are being initiated to measure the binding of chemical mutagens and carcinogens in the goal of a molecular understanding of the molecular basis of mutagenesis.

Drs. Amacher, Elliott and Lieberman are studying DNA repair synthesis in a variety of mammalian cell lines after exposure to acetylaminofluorene. While all cell lines remove substantial amounts of the bound mutagen, there are differences in rates of repair which are being further studied. In conjunction with this Dr. Lieberman is collaborating with Dr. Gary Stein of the University of Florida to study nuclear protein synthesis in cultured human cells after exposure to chemical mutagens. Repair induced by UV and acetylaminofluorene progresses without new histone or acidic protein synthesis.

Dr. Huang is developing a gene mutation detection system in human diploid fibroblasts. Forward mutations will be detected at a sex-linked locus (HGPRT) and an autosomal locus (AGPRT). Drs. Huang, Elliott and Ishii will also examine mutagenesis and *in vitro* transformation in the same cell lines to determine the relationship of mutagenesis to the oncogenic process.

Dr. Lieberman is collaborating with Dr. Darrell Stafford of the University of North Carolina in a study to isolate the ribosomal DNA cistron from human cells.

Studies in the Biochemical Genetics Section utilize a variety of approaches to develop new assay systems to detect gene mutations in mammalian somatic and germ cells. Drs. Valcovic and Malling have developed a biochemical specific locus test based on electrophoretically detectable enzyme loci. The system can detect mutations that result in either a non-functional enzyme or a functional protein with altered electrophoretic mobility. Results to date from a ⁶⁰Co irradiation experiment indicate that the assay is at least as sensitive as the morphological specific locus system. The advantages of the new system are (1) the number of loci per animal tested can be easily increased, (2) since many of the enzymes are also in the human population, it will provide a base-line for monitoring of humans for increased mutation frequency. Drs. Mareschal and Valcovic are also investigating the heat-stability of mouse enzymes to determine the utility of this endpoint as an indicator for induced mutation. It is possible that this could be incorporated into the electrophoresis assay and, hence, further increase the number of genetic loci tested.

Considerable progress has been obtained by Drs. Mitra and Malling in the development of a mutation detection system in mammalian sperm. Such a system is important not only for determining mutagenic activity of environmental chemicals, but also for monitoring human males in high hazard industrial situations. Sperm cells which stain under selective conditions would be presumptive mutants. Test conditions have been defined for three enzymes and validation studies are underway.

In a correlative area Dr. Maier and Dr. Malling are developing a system using a mammalian testes microsomal activation system. This is similar to the liver microsome system with bacterial testers which is in widespread use but offers the advantage of using testicular tissue; the crucial tissue of concern for hereditary mutations.

Successful refinement of the liver microsomal activating system coupled with bacterial tester strains has been utilized to initiate a program using a computer programmed mass screening system. This project will attempt to provide standardized protocols and computerized control of tests and data evaluation.

During the past year studies in the Mammalian Genetics Section have focused on the comparative sensitivities of different mouse strains as well as different routes of injection. In one project, Dr. William Sheridan is comparing the induction of dominant lethal mutations induced in a wide variety of inbred strains of mice after treatment with a given dose of ethyl methane-sulfonate (EMS). The objective of this work is to develop a battery of tester strains ranging from very sensitive to very resistant which will better mimic

the range of variation that we can expect in the heterogeneous human population. In a closely related line of work, Dr. Eugene Soares is comparing the effect of different routes of ingestion of EMS in two inbred strains and one hybrid strain. A comparison of the levels of dominant lethality found will be performed to determine the optimal conditions for the detection of the genetic activity of this compound.

In another line of work Dr. Sheridan is studying the effects of dietary deficiencies on the sensitivity of the mouse to induction of heritable translocations. Because a significant portion of the human population is suffering nutritional insufficiency, it is important to determine whether they are particularly more sensitive to mutagenesis from environmental chemicals.

A study to detect induced genetic changes in both somatic and germ cells is being undertaken by Drs. Soares. Both lymphocytes and sperm from chemically treated males will be examined for chromosomal aberrations. The aim of this approach is to use somatic cell mutagenesis as a predictor for germ cell effects. The success of this program could greatly reduce the cost and time of mammalian mutagenicity testing.

A new project initiated by Drs. Burki and Sheridan will study chromosome aberrations in early mouse embryos after mutagenic treatment of parents. While dominant lethal effects have been induced by several chemicals, the genetic causes have not been elucidated. This research will seek to determine the relative contribution of chromosome aberrations to this class of dominant lethal events.

Studies in the Population Genetics and Epidemiology Section are in progress to compare the spontaneous mutation rates in natural and laboratory environments. Sex-linked recessive lethals in *Drosophila* sperm obtained from both wild and laboratory reared males indicate no difference in mutation rate. Another line of work in collaboration with scientists in the Environmental Biometry Branch and North Carolina State University is analyzing changes in gene frequency in response to exposure to various environmental chemicals. These experiments involve monitoring gene frequencies of experimental *Drosophila* populations as they are exposed to various toxic environmental chemicals.

Dr. Gillespie, with Dr. Crump of the Environmental Biometry Branch, is developing mathematical models to describe the effect of migration on gene frequencies. This program attempts to answer basic questions about the role of geographic distribution of populations and migration upon the establishment and maintenance of genetic variability.

Efforts to establish low dose risk estimates from animal carcinogenesis experiments are being carried out by Drs. Langley and Hoel (Environmental Biometry Branch). Most laboratory studies use high doses of chemical carcinogens; and since humans may more often be exposed to low doses, it is important to determine low dose effects. This approach uses various models of carcinogenesis to generate dose response curves for low dose extrapolation.

Collaborative Research Program - In the Collaborative Research Program two new contracts were initiated in FY74 to detect gene mutations in somatic cells and germ cells in mammals. In the first contract at the University of Washington an attempt is being made to develop a simple system to measure point mutations in readily accessible human somatic cells. This assay is based on changes in the expected frequency of human red blood cells containing fetal hemoglobin rather than adult hemoglobin. Specific antisera are being developed to detect mutations which produce further A₂ or G₂ fetal hemoglobin. By conjugating the two antisera with different colored fluorescent dyes, mutant RBC's will fluoresce in only one color rather than two. In another contract at the Jackson Laboratory progress has been made toward the development of a new mouse strain carrying multiple chromosome inversions to make it possible to detect gene mutations (recessive lethal mutations) in the chromosome regions covered by the inversions. One of the 19 inversions recovered during the past year has been used in tests to detect x-ray induced recessive lethal mutations to demonstrate the general utility of this approach.

A second contract with the Jackson Laboratory involves the incorporation of 13 additional mouse enzyme alleles into the C57BL/6J strain. This will directly complement the electrophoretic assay system developed at our Institute and will significantly enhance the sensitivity of the system to detect biochemical specific locus mutations.

In another contract at the University of Wisconsin an assay is being designed to assay the induction of gene mutations at two different loci, HGPRT and AGPRT. During the past year, dose response curves have been obtained after treatment with two potent mutagens and carcinogens, MNNG and nitrosofluorene. The genetic and biochemical characterization of over 100 independently induced and spontaneous mutant cell lines has shown that the base-analog resistant mutants obtained with this assay are stable genetic variants and not transient phenocopies of some sort.

We have continued to support a contract at Miles Laboratory to perform the genetic analysis of ad-3 mutants recovered from experiments on haploid strains of two-component heterokaryons. During the past two years we have also been using repair-deficient strains of *Neurospora*, as the genetic characterization performed in this manner has shown striking qualitative differences between wild-type and excision-repair deficient strains.

By interagency agreement, we are supporting base-line studies to determine the mutagenicity of airborne chemical pollutants and other gases used by industry. Various clones of the plant *Tradescantia paludosa* are being developed to select one particularly sensitive clone for assays of the genetic activity of weak mutagens. Field studies are being planned during FY76 in collaboration with NERC-EPA to compare the effects of exposure to the same airborne pollutants under laboratory and field conditions.

During the past year we have also continued to support EMIC (Environmental Mutagen Information Center) by interagency agreement at the Oak Ridge National Laboratory. This center has over 15,500 bibliographic entries in its data banks and contains as a unique world-wide resource for information in the area of environmental chemical mutagenesis.

NATIONAL PROGRAMS

To develop better coordination in environmental mutagenesis and to provide perspective for the intramural scientific staff in problem definition and resolution in the rapidly developing field, a variety of mechanisms have been developed.

IPEM - (DHEW Interagency Panel on Environmental Mutagenesis, chaired by Dr. F.J. de Serres, Chief, Environmental Mutagenesis Branch, NIEHS) Intramural staff members have continued to hold meetings with at least 20-30 representatives from DHEW and other government agencies to develop better communication within DHEW as well as between other agencies with programs in environmental mutagenesis. The Panel meets monthly for discussion, and minutes are recorded to provide the basis for furthering communication within the various member institutes and agencies. The minutes of all meetings are included in a yearly report to the parent DHEW Committee to Coordinate Toxicology and Related Matters (chaired by Dr. D.P. Rall, Director, NIEHS).

Workshops and Conferences - In collaboration with the Pharmacology Branch, a small workshop was held on January 13-14, 1975, to discuss the general problem of *in vitro* metabolic activation and to plan a conference on this and related problems early in CY76.

Collaborative Programs with Other Institutes within DHEW and with Other Government Agencies

1. NCI - In collaboration with staff scientists at NCI, a collaborative research program has been developed to determine the correlation between carcinogenesis and mutagenic activity of chemical carcinogens and non-carcinogenic structural analogs. This has also involved development of mechanisms to review and evaluate performance of the contractors as well as the planners of workshops to evaluate the accomplishments of the overall program.

2. FDA - In collaboration with staff scientists in the Bureau of Foods, NIEHS staff scientists have participated in the successful development (during FY74) and review (during FY75) of collaborative research programs to perform mutagenicity tests on food additives.

3. EPA - In collaboration with staff scientists in the Office of Toxic Substances, NIEHS staff scientists have participated in the development of collaborative research programs to perform mutagenicity tests on pesticides.

4. NSF and Other Agencies - Staff scientists have participated in the review of proposals for contract work and in site visits to select suitable contractors for research in the area of mutagenicity testing.

Programs with the Environmental Mutagen Society (President - F.J. de Serres, Chief, Environmental Mutagenesis Branch, NIEHS) - During the past year, as a result of the work on Committee 17, a major position paper has been prepared and published (Science 187:503-514, 1975) to alert the scientific community and the general public about the hazards to man resulting from exposure to genetically active chemicals in our environment and the feasibility and necessity for mutagenicity screening.

Collaborative Studies on Genetically Active Environmental Chemicals - During FY74 a study was started to evaluate more thoroughly through tests on other laboratory organisms the mutagenicity of hycanthone and lucanthone, two drugs used to treat schistosomiasis. Most of the work has been completed during FY75, but the assays on human cells in culture may not be completed until FY76. In addition, in FY75 other collaborative studies were started to evaluate AF-2 (a nitrofurantoin derivative used extensively as a food preservative in Japan), commercially available hair dyes and anesthetic gases. The case study approach is being used in such cases to help us better understand the problems associated with evaluating the relative risk to man from different classes of genetically active environmental chemicals.

Program Reviews at Other National Laboratories with Major Programs in Environmental Mutagenesis - To provide an opportunity to develop better communication, collaboration and definition of problem areas in environmental mutagenesis, a program was initiated in FY75 to site visit other laboratories. These have included the Oak Ridge National Laboratory, Brookhaven National Laboratory and Lawrence Livermore Laboratory. In the latter case an extensive review of the projects was given by the Lawrence Livermore Laboratory staff members to the National Institute of Environmental Health Sciences staff members. The further development of such program reviews will facilitate development of complementary programs of research within each research group and facilitate initiation of collaborative research projects between staff scientists.

INTERNATIONAL PROGRAM

Staff members are associated with a wide variety of international programs with individual countries as well as with international agencies and programs.

US-Japan Cooperative Medical Sciences Program - In this program the U.S. Environmental Panel (Chairman, F.J. de Serres, Chief, Environmental Mutagenesis Branch, NIEHS) has organized two major workshops and conferences. The first conference on "Monitoring the Problem of Mutagenesis and Carcinogenesis in Man" was held on August 6-7, 1974, in Tokyo, Japan, in connection with the US-Japan Cooperative Medical Sciences Program. (Presentation of case studies on environmental and occupational carcinogens and mutagens. Review of laboratory tests for carcinogens and mutagens. Discussion of monitoring systems to evaluate carcinogenic and mutagenic effects.) A workshop on the mutagenicity of chemical carcinogens was held in Honolulu, Hawaii, on December 9-11, 1975, (Review status of mutagenicity data from tests of 152 chemical carcinogens and non-carcinogenic structural analogs and other non-carcinogenic chemicals).

US-USSR Environmental Protection and Environmental Health Agreements - In connection with the Environmental Protection Agreement staff members were responsible for the development of the Workshop on Basic and Practical Approaches to Environmental Mutagenesis and Carcinogenesis on April 28-30, 1975, held in New York City, New York. (Presentation of case studies of environmental carcinogens and mutagens, Techniques in population monitoring and epidemiology in environmental mutagenesis and carcinogenesis. Evaluation of population risk)

US-Germany Life Sciences Program - In connection with a new agreement with the Federal Republic of Germany, a collaborative effort will be made in mutagenesis and carcinogenesis by the National Institute of Environmental Health Sciences and the Gesellschaft für Strahlen-und Umweltforschung MGH, Munich. The initial program in this area will be to develop a better method for determining gene mutations in germ cells using electrophoretic techniques.

International Workshops - Staff members were involved in the planning of two major workshops during FY75. The first was held at the Bhabha Atomic Research Center in Bombay, India, from September 30 to October 11, 1974. This was a training course, primarily for Indians, to acquaint participants with the principal methods of mutagenicity testing.

The second meeting was a workshop on International Coordination of Environmental and Chemical Mutagenesis Studies held on May 13, 1975, in Miami Beach, Florida (discussion of international workshops and conferences, development of better methods to evaluate risk from exposure to environmental mutagens, development of mechanism for training, developing effective legislation for regulation of environmental mutagens).

Collaboration with IARC (Lyon) - During FY75 a new program has been started which involves participation between the National Institute of Environmental Health Sciences staff scientists, EMIC personnel and IARC scientists to translate mutagenicity data of known chemical carcinogens. Staff members are also participating in a series of workshops organized by the IARC staff to evaluate the correlation between carcinogenic and mutagenic activity and the possibility of using short-term tests for mutagenic activity to predict potential mutagenic and carcinogenic activity in man.

INFORMATION EXCHANGE

EMIC (Environmental Mutagen Information Center) - The Institute has continued to support the work of EMIC to collect, organize and disseminate information on environmental chemical mutagens. The Center not only processes requests for information from various government agencies, but is now becoming well known abroad. During the past year, EMIC's data file reached 15,500 bibliographic entries.

Journals - Staff members serve as associate editors, assistant editors or members of editorial boards of Mutation Research, Cancer Research, The Journal of Toxicology and Environmental Health Perspectives. This makes it possible not only to promote publication of papers dealing with various research areas of environmental mutagenesis but also to promote development of new methods for publication. Staff members were instrumental in development of two new supplements in Mutation Research during FY75: (1) Reviews in Genetic Toxicology and (2) Testing in Genetic Toxicology. The former section will enable publication of literature surveys and evaluation of data on a given chemical or group of chemicals. The latter will provide a mechanism for rapid publication of positive and negative data from routine screening tests on environmental chemicals.

TRAINING

The great shortage of scientists to do research in the area of environmental mutagenesis both in the United States and abroad has provided incentive for staff scientists to develop a training program at both the predoctoral and postdoctoral levels. During the past year, we have provided facilities for research for three predoctoral students from the University of North Carolina at Chapel Hill. At the postdoctoral level, staff scientists include six staff fellows and seven visiting fellows and one visiting scientist. In the visiting program, postdoctoral fellows in training have come from Switzerland (2), Belgium (1), Federal Republic of Germany (1), Australia (1), Japan (3), England (1), and India (1).

In conclusion, during the past year we have been successful in recruiting key personnel for our intramural program, have started cooperative programs with other Branches in the Institute, have coordinated program development of the Branch with complementary programs in other Federal agencies, and have established lines of communication with newly developing scientific organizations for environmental mutagenesis abroad. Along with the organization of workshops and conferences, these approaches will be used to stimulate research and development in this rapidly developing area of environmental health science.

Project No.: Z01 ES 60001-01 EMB
1. Environmental Mutagenesis Branch
2. Office of the Chief
3. Research Triangle Park, N. C.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

PROJECT TITLE: "Mutation Induction in Repair-Deficient Two Component Heterokaryons of Neurospora crassa"

PREVIOUS SERIAL NUMBER: None

PRINCIPAL INVESTIGATOR: Frederick J. de Serres, Ph.D.

OTHER INVESTIGATORS: None

COOPERATIVE UNITS: None

MAN YEARS

Total	1.2
Professional:	0.2
Other:	1.0

PROJECT DESCRIPTION

OBJECTIVES: Previous studies (NIEHS-MB-003) have shown that the process of gene mutation is under genetic control and that both marked quantitative and qualitative differences can be found in such strains (when compared with the standard wild-type strain) with regard to mutation induction at specific loci. In two excision repair-deficient strains, upr-1 and uvs-2, enhanced mutant recovery was found after UV or X irradiation -- and more recently after MNNG treatment (NIEHS-MB-Inoue's report of this year). Genetic characterization of the UV-induced ad-3B mutants induced in the repair-deficient strains showed that they were qualitatively different from the UV-induced ad-3B mutants in our standard wild-type strain (NIEHS-MB-003).

In a two-component heterokaryon, mutations in the ad-3 region result both from point mutation and chromosome deletions; the latter class is haplo-lethal and cannot be recovered in experiments on haploid strains. Various studies with variation in dose-rate with x-rays, photoreaction after UV, etc., have shown that the lesions giving rise to these two classes of mutations are qualitatively different. The objective of the present study is to determine the effect of upr-1 and uvs-2 on the induction of irradiation-induced chromosome deletions in the ad-3 region.

METHODS EMPLOYED: Both components of heterokaryon 12 (our standard wild-type strain) were crossed to either upr-1 or uvs-2, to enable us to construct new two-component heterokaryons which are homozygous for either mutation. These

two new strains (which are heterozygous for ad-3A and ad-3B) were then used in a series of x-ray experiments to determine the effect of each repair-deficient mutation on spontaneous and x-ray-induced mutation in the ad-3 region.

MAJOR FINDINGS: The results of mutation-induction experiments showed that spontaneous mutation in the ad-3 region in the new heterokaryons, homozygous for either upr-1 or uvs-2 was not different from heterokaryon 12. However, after X-irradiation, both strains required at least a 5- to 8-fold reduction in exposure to give similar forward-mutation frequencies as in the standard wild-type strain.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Recent studies by others have shown that in man xeroderma pigmentosa patients are deficient in excision repair. Cells of such patients in culture show enhanced inactivation after irradiation as well as enhanced induction of gene mutations and chromosome aberrations. Only limited analysis of the gene mutations can be made to determine whether they are qualitatively different from those found in experiments on normal human cells in culture. In *Neurospora* the ad-3 mutations recovered can be readily characterized by a series of simple genetic tests.

PROPOSED COURSE: Samples of ad-3 mutants induced by x-rays in both repair-deficient strains have been reserved for genetic analysis. This analysis will enable us to determine whether the enhanced recovery of ad-3 mutants in such strains is comparable for both point mutations and chromosome deletions.

KEYWORD DESCRIPTORS: Repair deficient strains, *Neurospora crassa*, mutation induction, specific loci, x-rays, ad-3A, ad-3B.

Project No.: Z01 ES 60002-03 EMB

1. Environmental Mutagenesis Branch
2. Microbial & Plant Genetics Section
3. Research Triangle Park, NC

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

PROJECT TITLE: "Mutagenicity and Mutagenic Specificity of Environmental Agents in Neurospora crassa"

PREVIOUS SERIAL NUMBER: NIEHS-EM-004

PRINCIPAL INVESTIGATOR: Tong-man Ong, Ph.D.

OTHER INVESTIGATOR: None

COOPERATING UNITS: Miles Laboratory, Elkhart, Indiana

MAN YEARS

Total:	1.1
Professional:	0.1
Other:	1.0

PROJECT DESCRIPTION

OBJECTIVES: Many synthetic nitrofurans and related compounds are being used as chemotherapeutic agents in the treatment of human disease. Some others are being used as antibacterial food additives and preservatives. These compounds, therefore, are very closely related to human health. Our previous studies with nitrofurans have shown that a chemotherapeutic agent, a food additive and an antischistosomal agent are mutagenic in Neurospora crassa. Our studies also show that a NO₂ group at the C5 position and a particular side chain at the C2 position are necessary for the mutagenic activity of nitrofurans. It would be of interest to determine whether other chemotherapeutic agents are mutagenic in Neurospora crassa to verify function-structure relationships of nitrofurans and to determine the type of genetic alterations caused by chemotherapeutic agents.

METHODS EMPLOYED: The ad-3 test system of Neurospora crassa developed by F. J. de Serres is used in this study. In general, mutation induction experiments could be described as follows: conidia from a genetically marked two-component heterokaryon are harvested and are treated with different concentrations of nitrofurans in sodium-phosphate buffer, pH 7, at 30°C for two hours in a water bath shaker in the dark. At the end of the treatment chemicals are decanted and conidia are washed by centrifugation. The treated and untreated conidia are inoculated into 10 liters of Westergaard's medium with the appropriate supplements. After 7 days incubation in the dark at 30°C, the frequency of purple ad-3 mutants are determined in each treatment. The isolated ad-3 mutants are then characterized by a series of genetic tests.

MAJOR FINDINGS: The mutagenicity of 11 nitrofurans have been studied with the ad-3 test system of *Neurospora crassa*. Nitrofurazon, AF-2, SQ18506, FANFT and H-193 were all found to be mutagenic. The mutagenic activity of H-193 appears to be less than that of the closely related antibacterial food additive, AF-2.

Metronidazole, a well-known antitrichomonal drug used in the treatment of women, is not mutagenic in *Neurospora crassa* when conidia are treated. This compound, however, is highly mutagenic in the growing vegetative cultures of *Neurospora crassa*. It seems that the mutagenic activity of metronidazole requires metabolic activation. Genetic characterization of ad-3 mutants induced by nitrofurans is still in process. Preliminary data indicate that nitrofurans predominantly induce base-pair substitution mutations.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Many nitrofurans are used as antibacterial drugs or as antibacterial food additives or food preservatives. Studies on the mutagenic activities of nitrofurans could provide information in regard to the potential mutagenic hazards of nitrofurans in humans. Furthermore, studies on the mutagenicity and the chemical structure relationships of nitrofurans could lead to a better understanding of the mutagenicity and other biological activities of nitrofurans such as antibacterial, antischistosomal and carcinogen activities and might lead to a production of non-mutagenic compounds that could be used as food additives or as drugs for the treatment of disease in man.

PROPOSED COURSE: Future studies will continue on those agents that are closely related to human health and those structurally related compounds. Future studies will also be carried out to determine types of genetic alterations caused by environmental agents and to determine the relationship between their mutagenic activities and their chemical structure.

KEYWORD DESCRIPTORS: Environmental agents.

PUBLICATIONS

Ong, T. and Shahin, M. M.: Mutagenic and recombinogenic activities of the food additive furylfuramide in eukaryotes. Science 184:1086-1089, 1974.

Project No.: Z01 ES 60003-02 EMB

1. Environmental Mutagenesis Branch
2. Microbial & Plant Genetics Section
3. Research Triangle Park, NC

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

PROJECT TITLE: "Mutagenic Activity of Chemical Carcinogens in Neurospora crassa"

PREVIOUS SERIAL NUMBERS: NIEHS-EM-005

PRINCIPAL INVESTIGATOR: Tong-man Ong, Ph.D.

OTHER INVESTIGATOR: Frederick J. de Serres, Ph.D.

COOPERATING UNITS: Miles Laboratory, Elkhart, Indiana

MAN YEARS

Total:	.3
Professional:	.3
Other:	.0

PROJECT DESCRIPTION

OBJECTIVES: Recent studies on the mutagenicity of chemical carcinogens in various test systems have shown that there is a positive correlation between mutagenicity and carcinogenicity. Our previous studies in Neurospora crassa show that carcinogenic compounds in laboratory mammals are also mutagenic in N. crassa and that non-carcinogenic compounds are non-mutagenic. Our studies also indicate that potent carcinogens induce predominantly base-pair substitution mutations. It is necessary to extend our studies to other carcinogenic compounds to determine whether those compounds are also mutagenic in Neurospora and to determine whether those compounds induce base-pair substitution mutations.

METHODS EMPLOYED: The ad-3 test system of N. crassa developed by F.J. de Serres is used in this study. Conidia from a genetically marked two-component heterokaryon are treated with chemical carcinogens. The treated and untreated conidia are analyzed for the presence of ad-3 mutants by the direct method. Based on the background counts and the number of mutants, the survival and the mutation frequency are determined. The ad-3 mutants isolated from forward mutation experiments are made homokaryotic for the adenine requirements. The genotype and the complementation pattern of each mutant is determined by heterokaryon tests with various strains used as testers. Mutants are then tested by means of dikaryon and trikaryon tests to determine the frequency of chromosomal deletions.

MAJOR FINDINGS: Genetic analysis of ad-3 mutants induced by chemical carcinogens show that potent chemical carcinogens such as 4NQO, 4HAQO, PMMT, aflatoxins and 2AN cause predominantly base-pair substitution mutations in N. crassa. 4NQO and its hydroxylated compound, 4HAQO, appear to induce identical genetic alterations.

Dimethylnitrosamine, as well as 3 carcinogenic nitrofurans, are found to be mutagenic in N. crassa. The mutagenic activity of DMN, however, requires metabolic activation. In these studies we also demonstrate that in vitro metabolic activation techniques can be incorporated into ad-3 test systems.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Studies on the mutagenicity of chemical carcinogens in N. crassa might provide a system that could be used for screening carcinogenic chemical agents in the human environment. Furthermore, the studies on the spectrum of genetic alterations, caused by chemical carcinogens in N. crassa might provide information in regard to the relationship between carcinogenesis and mutagenesis of chemical agents.

PROPOSED COURSE: Future study will be extended to other chemical carcinogens including those compounds in which metabolic activation is necessary for the expression of their carcinogenic activity.

KEYWORD DESCRIPTORS: Chemical carcinogens

PUBLICATIONS

Ong, T., Matter, B. E. and de Serres, F. J.: Genetic characterization of adenine-3 mutants induced by 4-nitroquinoline 1-oxide and 4-hydroxyaminoquinoline 1-oxide in Neurospora crassa. Cancer Research 35:291-295, 1975.

Project No.: Z01 ES 60004-02 EMB

1. Environmental Mutagenesis Branch
2. Microbial & Plant Genetics Section
3. Research Triangle Park, NC

PHS-NIH

Individual Project Report
July 1, 1974 through June 30, 1975

PROJECT TITLE: "Development of a Spot Test System in Neurospora crassa"

PREVIOUS SERIAL NUMBER: NIEHS-EM-006

PRINCIPAL INVESTIGATOR: Tong-man Ong, Ph.D.

OTHER INVESTIGATORS: None

COOPERATING UNITS: None

MAN YEARS

Total:	1.4
Professional:	0.4
Other:	1.0

PROJECT DESCRIPTION

OBJECTIVES: The adenine-3 (ad-3) forward mutation test system in Neurospora crassa developed by F. J. de Serres is one of the best systems in studying chemical and physical mutagenesis in eukaryotic microorganisms. With this test system, recessive lethal mutations resulting from either chromosome deletions or point mutations can be recovered at the ad-3 region. The isolated mutants can be characterized by a series of genetic tests and the type of genetic alterations at the molecular level can be determined. The limitations of this test system, however, are that one cannot screen for the mutagenicity of a large number of chemicals in a short period of time and that not every laboratory can use this test system. It seems, therefore, necessary to develop a spot test system in N. crassa and to search for different tester strains which can be used for detecting specific types of genetic alterations such as base-pair substitutions and frameshift mutations. The development of a spot test in N. crassa will make the ad-3 test system a more complete and useful system for the detection and evaluation of the mutagenic activity of environmental agents.

METHODS EMPLOYED: Development of a spot test system involves two steps. The first step is establishing the optimal test conditions in N. crassa and the second step is finding the tester strains which can detect several different types of genetic alterations. Conidia from 7 day-old vegetative cultures of ad-3 mutants are harvested. A specific number of conidia are added into 2 ml of cover medium (Fries' minimal medium). The mixture of conidia and cover medium is poured onto a plate containing 20 ml of Fries' minimal medium.

Samples of known chemical mutagens are placed on the center of the plate. After 6 days incubation at 30°C, revertants from each plate are recorded. The results from different test conditions are compared. The conditions that give optimal results will be used for the future spot tests.

In the second step, reverse mutations of a large number of ad-3 mutants from different mutagenic origins are tested with several known chemical mutagens (MNNG, hydroxylamine, 4NQO, ICR-170, AF-2, EMS, etc.) by spot tests. Mutants which exhibit the best responses to the mutagens are selected as tester strains.

MAJOR FINDINGS: We have established optimal conditions for the spot test and have selected 8 strains from hundreds of mutants as preliminary tester strains. The mutagenicity of MNNG, ICR-170, 4NQO, AF-2, SQ18506, EMS, EI, BP, FANFT, H-193, N-acetoxy-AAF and N-hydroxy-AAF can all be detected by the simple test system.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Development of a spot test system will provide a simple, economical and quick system in eukaryotes for the detection and evaluation of the mutagenic activity of chemical agents in the human environment.

PROPOSED COURSE: Future efforts will limit the number of testers to 4. A UV-sensitive marker will be introduced to the tester strains to increase their mutagenic sensitivity. In vitro metabolic activation will also be incorporated into the test system.

KEYWORD DESCRIPTORS: Spot test system

1. Environmental Mutagenesis Branch
2. Microbial & Plant Genetics Section
3. Research Triangle Park, NC

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

PROJECT TITLE: "Mutagenic Activity of Antischistosomal Drugs and Their
Analogues in Neurospora crassa"

PREVIOUS SERIAL NUMBER: NIEHS-EM-007

PRINCIPAL INVESTIGATOR: Tong-man Ong, Ph.D.

OTHER INVESTIGATOR: Frederick J. de Serres, Ph.D.

COOPERATING UNITS: None

MAN YEARS

Total:	0.4
Professional:	0.4
Other:	0.0

PROJECT DESCRIPTION

OBJECTIVES: Hycanthone, lucanthone and niridazole, chemotherapeutic agents in the treatment of schistosomiasis, are attracting wide concern because of their potential carcinogenic and mutagenic hazards to treated individuals in the human population. Our previous studies have shown that hycanthone, lucanthone and their indazole analogs are mutagenic in Neurospora crassa. Niridazole is not mutagenic under the condition that conidia were treated. It would be of interest to test the mutagenicity of niridazole in growing vegetative cultures of N. crassa and to determine the type of genetic alterations caused by hycanthone, lucanthone and their indazole analogs.

METHODS EMPLOYED: The ad-3 test system of N. crassa is used in these studies. Conidia from a genetically marked, two-component heterokaryon are harvested and are treated with different concentrations of antischistosomal agent in sodium-phosphate buffer, pH 7, at 30°C for 2 hours in a water bath shaker in the dark. At the end of the treatment, chemicals are decanted and conidia are washed by centrifugation. The treated and untreated conidia are inoculated into 10 liters of Westergaard's medium with the appropriate supplements. After 7 days incubation in the dark at 30°C, the frequency of purple ad-3 mutants are determined in each treatment. The isolated mutants are made homokaryotic for the adenine requirements. The genotype and the complementation patterns of each mutant are determined by growing each mutant with each complementation tester. Based on the complementation patterns, mutants can be classified as noncomplementing, polarized complementing and nonpolarized complementing mutants. The mutants are further tested with dikaryon and

and trikaryon tests to determine the frequency of chromosomal deletions.

MAJOR FINDINGS: The mutagenic activities of hycanthone, lucanthone and their indazole analogs in N. crassa indicate that compounds with a methyl group at the C4 position are more mutagenic than compounds with a methanol group; 6-chloro-indazole analogs are more mutagenic than nonchlorinated analogs. The data from N. crassa show that there is no correlation between mutagenicity and antischistosomal activity.

Niridazole is not mutagenic in N. crassa conidia. This compound is mutagenic, however, when the vegetative cultures are treated. This study suggests that metabolic activation is necessary for the expression of the mutagenic activity of niridazole. Preliminary results of genetic analyses of ad-3 mutants induced by hycanthone and its analogs indicate that these compounds predominantly induce multilocus deletions.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Studies on the mutagenicity of niridazole, hycanthone, lucanthone and their structural analogs are essential and important, since hycanthone and niridazole are widely used for treatment of schistosomiasis in the human population. It is important to determine the potential mutagenic hazard of these compounds to the treated individuals. Studies on the relationship between structures and mutagenic activities of hycanthone and its analogs might lead to a production of non-mutagenic compounds that can be used for the treatment of schistosomiasis. This analysis will provide some experience with regard to structure-function relationships which will be important for future work with mutagenic environmental chemicals. We need to know whether we can develop structural analogs of desirable, but mutagenic chemicals that will retain the desired pharmacological properties, but not the undesirable mutagenic properties.

PROPOSED COURSE: Future studies on the mutagenic activity of hycanthone and its analogs will be focused on the mechanism of mutation induction by these compounds and niridazole in N. crassa.

KEYWORD DESCRIPTORS: Antischistosomal drugs.

Project No.: Z01 ES 60006-01 EMB

1. Environmental Mutagenesis Branch
2. Microbial & Plant Genetics Section
3. Research Triangle Park, NC

PHS-NIH

Individual Project Report

July 1, 1974 through June 30, 1975

PROJECT TITLE: "Mutagenicity and Mutagenic Specificity of Chemical Carcinogens in UV-Sensitive Strains of Neurospora crassa"

PREVIOUS SERIAL NUMBER: None

PRINCIPAL INVESTIGATOR: Hirokazu Inoue, Ph.D.

OTHER INVESTIGATORS: Tong-man Ong, Ph.D. and Frederick J. de Serres, Ph.D.

COOPERATING UNITS: None

MAN YEARS

Total:	1.2
Professional:	1.2
Other:	0.0

PROJECT DESCRIPTION

OBJECTIVES: Many studies, in bacteria as well as in other microbial organisms, have shown that some of the repair-deficient strains are more mutable than the wild type. In studies with the ad-3 test system of Neurospora crassa, Dr. de Serres and his coworkers showed that the repair-deficient strains, upr-1 and uvs-2, are more mutable than wild type after UV or gamma-ray irradiations. It has also been shown by de Serres that the spectra of genetic alterations caused by UV in repair-deficient strains are different from that caused by UV in the wild type. It is of interest, therefore, to study and compare the mutagenic sensitivity of repair-deficient and wild-type strains to various chemical carcinogens and to compare the spectra of genetic alterations caused by these carcinogenic agents in the UV-sensitive and the wild-type strains.

METHODS EMPLOYED: The ad-3 test system of Neurospora crassa developed by F. J. de Serres is used in this study. Conidia from UV-sensitive strains or wild type are treated with chemical carcinogens. The treated and untreated conidia are analyzed for the presence of ad-3 mutants by the direct method. The isolated ad-3 mutants from forward mutation experiments are made homo-karyotic for the adenine requirement. The genotype and complementation pattern will be determined by complementation tests.

MAJOR FINDINGS: The results of the mutation induction experiments with MNNG indicate that both uvs-3 and uvs-5 strains are slightly less mutable than wild type whereas uvs-6 show a sensitivity similar to wild type. By contrast,

however, upr-1 is two times and uvs-2 is five times more sensitive than wild type. Present data show that the differences in the responses of the UV-sensitive strains to MNNG from the wild-type strain seem to be most similar to the differences observed in their response to UV with exception of uvs-3 which was stable to mutation-induction after UV. The response of these strains to MNNG is different from their response to gamma-ray irradiation.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: This study shows that the repair-deficient strains, upr-1 and uvs-2, are not only more mutable than wild type by UV or gamma-ray irradiation but also more sensitive to the chemical carcinogen, MNNG. If these strains are also more sensitive than wild type to other chemicals, the use of repair-deficient strains would facilitate the detection of mutagenic activity in various environmental agents.

PROPOSED COURSE: Studies on the mutagenicity and mutagenic specificity with other carcinogens will be performed in these repair-deficient strains and the role of repair ability in mutagenesis and carcinogenesis will be investigated.

KEYWORD DESCRIPTORS: Chemical carcinogens

Project No.: Z01 ES 60007-01 EMB

1. Environmental Mutagenesis Branch
2. Microbial & Plant Genetics Section
3. Research Triangle Park, NC

PHS-NIH

Individual Project Report
July 1, 1974 through June 30, 1975

PROJECT TITLE: "The Relationship Between Self-Inhibitors of Germination and Mutation Induction in Neurospora crassa"

PREVIOUS SERIAL NUMBER: None

PRINCIPAL INVESTIGATOR: Barry R. Scott, Ph.D.

OTHER INVESTIGATORS: None

COOPERATING UNITS: None

MAN YEARS

Total:	.2
Professional:	.2
Other:	.0

PROJECT DESCRIPTION

OBJECTIVES: Recent studies with Aspergillus nidulans have shown that marked differences in induced mutation frequencies in apparently replicate exposures to mutagens are mainly due to the presence of varying amounts of self-inhibitors of germination in conidia at the time of mutagenic treatment. With some mutagens removal of these compounds has also affected the qualitative mutational response of thirteen genes which may be simultaneously studied in the organism, Aspergillus nidulans. It would be very interesting to determine whether such substances are also present in the conidia of Neurospora crassa and to investigate the effect that these substances might have on the processes of mutation induction.

METHODS EMPLOYED: No information available because project has just been initiated (March 1975).

MAJOR FINDINGS: No information available because project has just been initiated (March 1975).

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND TO THE PROGRAM OF THE INSTITUTE: One of the greatest difficulties facing the establishment of test systems for routine quantitative mutagenicity testing is the heterogeneity of the test organism. Any procedure which reduced this biological heterogeneity increases the confidence with which the mutagenic data obtained with a test system can be meaningfully extrapolated to man. By controlling the amount of possible

germination inhibitory substances in *Neurospora* conidia, the degree of heterogeneity of any mutagenic test system which uses this organism is decreased. Isolation of the inhibitory substance might also be very useful in extending the treatment time which may be given to *Neurospora* in a host-mediated assay or microsomal activation situation, hence increasing the detection capability of *Neurospora*. Finally, such a study should also lead to a greater understanding of the basic processes involved in mutation induction.

PROPOSED COURSE: Initial studies will be directed toward detection and isolation of the possible self-inhibitory substances in the conidia of *Neurospora*. If such compounds are detected then the effect of these substances on mutation induction will be investigated.

KEYWORD DESCRIPTORS: Self-inhibitors of germination, qualitative mutational response, *Neurospora crassa*, extrapolated to man.

Project No.: Z01 ES 60008-01 EMB

1. Environmental Mutagenesis Branch
2. Microbial & Plant Genetics Section
3. Research Triangle Park, NC

PHS-NIH

Individual Project Report
July 1, 1974 through June 30, 1975

PROJECT TITLE: "Mutagenic Activity of Furo-Coumarins in Various Micro-organisms"

PREVIOUS SERIAL NUMBER: None

PRINCIPAL INVESTIGATOR: Barry R. Scott, Ph.D.

OTHER INVESTIGATORS: George R. Mohn, Ph.D. and Tong-màn Ong, Ph.D.

COOPERATING UNITS: None

MAN YEARS

Total:	.9
Professional:	.9
Other:	.0

PROJECT DESCRIPTION

OBJECTIVES: Furo-coumarins in combination with blacklight are being used as chemotherapeutic agents in the treatment of diseases in man such as Xeroderma pigmentosum and Psoriasis. These compounds are also commonly occurring in man's natural and artificial environments. The possible exposure of the human population to these compounds is, therefore, relatively high and cause for concern. Systematically, little information is known about the comparative mutagenic, carcinogenic and teratogenic effects of the furo-coumarins. It would be interesting to determine the comparative mutagenicity of one of the furo-coumarins, 8-methoxypsoralen (8-MOP), with various combinations of blacklight in a variety of microbial systems and to determine the type of genetic alteration caused by 8-MOP in each regime. In the later stages of the project the investigation will be extended to other furo-coumarins in order to determine whether there is a relationship between the mutagenicity and chemical structure of the furo-coumarins.

METHODS EMPLOYED: No information available because project has just been initiated (March 1975).

MAJOR FINDINGS: No information available because project has just been initiated (March 1975).

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND TO THE PROGRAM OF THE INSTITUTE:
Studies on the mutagenicity of the furo-coumarins are essential and important

since some of the compounds are widely used for treatment of Xeroderma pigmentosum and Psoriasis in human populations. The relationship between various microbial test systems could provide information on the potential mutagenic hazard of 8-MOP in humans. Furthermore, studies on the mutagenicity and the chemical structure of the furo-coumarins could lead to a better understanding of the mutagenicity and other biological activities of the furo-coumarins; this might result in either the production of non-mutagenic compounds that could be used for the treatment of diseases in man or treatment conditions which would reduce the exposure risk.

PROPOSED COURSE: Studies with various microbial test systems which detect mutation induction will be used in assessing three major types of treatment condition for mutagenesis. These are (a) 8-MOP without radiation, (b) 8-MOP plus low doses of radiation and (c) 8-MOP plus high doses of radiation.

Future studies will also be carried out to determine the types of genetic alterations caused by furo-coumarins as well as determining the relationship between mutagenic activity and chemical structure.

KEYWORD DESCRIPTORS: Furo-coumarins, Xeroderma pigmentosum, Psoriasis, comparative mutagenic effects, 8-methoxypsoralen

Project No.: Z01 ES 60009-01 EMB

1. Environmental Mutagenesis Branch
2. Microbial & Plant Genetics Section
3. Research Triangle Park, NC

PHS-NIH

Individual Project Report

July 1, 1974 through June 30, 1975

PROJECT TITLE: "Mutagenic Properties of Hycanthone and Some Analogs in Escherichia coli"

PREVIOUS SERIAL NUMBER: None

PRINCIPAL INVESTIGATORS: George R. Mohn

OTHER INVESTIGATORS: None

COOPERATING UNITS: None

MAN YEARS

Total:	.2
Professional:	.2
Other:	.0

PROJECT DESCRIPTION

OBJECTIVES: Since the demonstration of the mutagenic activity of the anti-schistosomal drug, hycanthone, in several microbial assay systems suggesting a potential mutagenic effect in humans treated with the drug, several analogs have been synthesized; some of them -- the chloroindazole derivatives -- still possess antischistosomal activity but have reduced mutagenic effects in Salmonella typhimurium. In this study the compounds, IA-3, IA-3-N-oxide, IA-4, and IA-4-N-oxide, are tested for mutagenicity in several genes in a strain of Escherichia coli and the results compared to those obtained in Salmonella typhimurium.

METHODS EMPLOYED: Because the compounds have already been shown to express direct mutagenic activities, only in vitro tests will be performed, mainly by incubating the indicator bacterial strain together with the test substance at 37° and plating on selective, mutation-detecting media. Both time-dependent and concentration-dependent experiments are to be made. The bacterial indicator is Escherichia coli K-12/343/113, a multipurpose strain (arginine-requiring, galactose-nonfermenting and methyltryptophan-sensitive) which allows the detection of several different types of mutations by plating aliquots of the bacterial population on different selective media.

The compounds are expected to induce mutations of the reading-frameshift type and will, as demonstrated in preliminary experiments, exert their mutagenic activity preferentially on dividing bacterial cells. Therefore, instead of using the traditional method with stationary cells, new methods will be

developed to determine the optimal conditions for mutagenesis with the compounds under investigation.

MAJOR FINDINGS: In preliminary experiments, it was shown that the systems detecting mutations to arg^+ , gal^+ and MTR resistance in E. coli are less sensitive to the mutagenic action of hycanthone than the his^+ mutation induction in strain S. typhimurium TA1538.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: This comparative study of the sensitivity of Salmonella and Escherichia to known mutagens will provide some indication whether structure-action relationships are similar in different bacterial species; they will allow further the choice of appropriate indicators for testing particular classes of compounds. Finally, should the compounds tested have reduced mutagenic activity compared to hycanthone as already shown in Salmonella, additional information would be obtained, indicating that these analogs are potentially less mutagenic in treated humans while retaining the desired therapeutic properties.

PROPOSED COURSE: After development of testing methods suitable for that class of compounds, further newly synthesized antischistosomal drugs may be tested.

KEYWORD DESCRIPTORS: Mutagenic activity, antischistosomal drug, hycanthone, IA-3, IA-3-N-oxide, IA-4, IA-4-N-oxide, bacterial indicator, Escherichia coli.

Project No.: Z01 ES 60010-01 EMB

1. Environmental Mutagenesis Branch
2. Microbial & Plant Genetics Section
3. Research Triangle Park, NC

PHS-NIH

Individual Project Report
July 1, 1974 through June 30, 1975

PROJECT TITLE: "Comparison of the Mutagenic Activity of Hair Dyes in Escherichia coli after Biotransformation through Isolated Rodent Liver Homogenates"

PREVIOUS SERIAL NUMBER: None

PRINCIPAL INVESTIGATOR: George R. Mohn

OTHER INVESTIGATORS: None

COOPERATING UNITS: None

MAN YEARS

Total:	.2
Professional:	.2
Other:	.0

PROJECT DESCRIPTION

OBJECTIVES: In recent reports, about 90% of commercial oxidative-type hair dye formulations were described as mutagenic in a reverse-mutations detecting strain of Salmonella typhimurium, the mutagenicity being expressed directly or after activation through rodent liver microsomes. The purpose of the present study is to compare these findings with those obtained in a strain of Escherichia coli where mostly forward mutations in several genes dispersed on the chromosome can be detected.

METHODS EMPLOYED: The indicator bacterial strain used in this study is Escherichia coli K-12/343/113. It is a multipurpose strain allowing the simultaneous detection of mutations in several different genes by plating aliquots of the bacterial population on different selective media. The mutational types detected include point mutations (e.g., those due to coding base-pair changes) as well as genetic events involving gene deletions and gross DNA regions. Mainly four systems are used: Reverse mutations from arg^{-56} (arginine deficiency) to arg^{+} and from nad^{-113} (nicotinic acid deficiency) to nad^{+} ; forward mutations from 5-methyltryptophan (MTR)-sensitivity to MTR-resistance and from galR^{S-18} (galactose regulatory gene) to gal^{+} . In the latter system, introduction of L-lysine into the medium will allow the detection of genetic events (e.g., deletions) extending between the genes galR and lysA on the E. coli chromosome.

To determine the influence of mammalian foreign compound metabolism on the genetic activity of the substances under study, the indicator bacteria are incubated together with extracts of mouse liver supplemented with cofactors. A further method of increasing the activity of liver preparation is to prefeed the animals to be used with enzyme inducers. Finally the mutagenicity of selected hair dyes will be tested in newly developed host-mediated assays where the indicator bacteria are inoculated into the blood stream of rodents. After mutagenic treatment the bacteria are removed from the liver where they have accumulated and are then tested for induced mutation frequencies.

MAJOR FINDINGS: In contrast to the results obtained in Salmonella typhimurium, the hair dyes so far tested in preliminary experiments show no detectable, or a rather weak, direct mutagenicity in Escherichia coli.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Comparative studies on the mutagenic activity of hair dyes are needed because of the wide use of these compounds in the human population. Such studies might lead to the detection of compounds with hair dyeing properties but lacking mutagenic effects. Furthermore, should the results of mutation experiments with E. coli be consistently negative in contrast to those obtained with S. typhimurium, a re-evaluation of the significance of mutations induced in specific bacterial strains will become necessary. Finally, a large-scale comparison of the mutagen sensitivities of Salmonella and Escherichia strains may facilitate the choice of appropriate bacterial indicators in routine mutagenicity screening tests.

PROPOSED COURSE: After experiments in vitro, tests will be made using liver homogenates of normal or microsomal enzymes induced mice. Further studies may include testing of newly developed hair dye analogs.

KEYWORD DESCRIPTORS: Hair dye, mutagenicity, activation through rodent liver microsomes, Escherichia coli, forward mutations in several genes.

Project No.: Z01 ES 60011-01 EMB

1. Environmental Mutagenesis Branch
2. Microbial & Plant Genetics Section
3. Research Triangle Park, NC

PHS-NIH

Individual Project Report

July 1, 1974 through June 30, 1975

PROJECT TITLE: "Development of New Host-Mediated Mutagenicity Tests"

PREVIOUS SERIAL NUMBER: None

PRINCIPAL INVESTIGATOR: George R. Mohn

OTHER INVESTIGATOR: Barry R. Scott

COOPERATING UNITS: None

MAN YEARS

Total:	.6
Professional:	.6
Other:	.0

PROJECT DESCRIPTION

OBJECTIVES: Since the demonstration that the mutagenicity of chemicals can be greatly altered by mammalian foreign compound metabolism before reaching the target -- somatic or germinal -- cells, several techniques have been developed to combine the advantages of microbial indicators of gene and gene segment mutations with parts of the mammalian metabolism. In the traditional host-mediated assay, the microbial indicators are inoculated into the peritoneal cavity of a murine host and recovered from there after treatment of the animal. Newly developed techniques tend to bring the indicator cells in closer contact to the organ where metabolic changes are most likely to occur, usually the liver. In principle this can be done by inoculating the indicator cells into the blood stream; after a short incubation time, a great proportion of the cells will be retained - alive - in the liver from where they can be isolated again and tested for mutation frequency. The purpose of this investigation is to develop techniques where the microbial indicators would remain in the blood stream for a longer period of time, so that mutagenic activities of metabolites within the blood stream of treated animals could be detected.

METHODS EMPLOYED: In order to prevent the phagocytosis of the indicator cells present in the blood stream of mice or rats, two approaches will be assayed: (1) Pretreatment of the rodent with substances which will either saturate the cells of the reticulo-endothelial system (e.g., carbon particles) or reduce the phagocytic activity (e.g., alkylating agents); (2) Use of microbial indicator cells which are resistant to the immunological host-defense mechanisms (e.g., Salmonella typhimurium instead of Escherichia coli). The

sensitivity of the techniques will then be tested by means of reference mutagens known to require mammalian metabolic activation.

MAJOR FINDINGS: Preliminary experiments have shown that some semi-rough strains of *Salmonella typhimurium* remain to a large extent within the blood stream of mice (approximately 2 hours) before invading sepsis occurs.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The importance of such techniques lies in the fact that they would provide the first possibility to follow the time-dependent appearance of mutagenic metabolites in the blood of treated, living animals. After calibration with reference mutagens, they could be incorporated easily into routine mutagenicity tests.

PROPOSED COURSE: Using microbes like bacteria or fungi as genetic indicators could lead in some rare cases to results which are due uniquely to microbial metabolism (and thus not relevant for mammals). These studies will, therefore, be extended to microorganisms in a growth phase lacking metabolism, e.g., spores and/or bacteriophages.

KEYWORD DESCRIPTORS: Mutagenicity of chemicals, mammalian foreign compound metabolism, host-mediated assay, microbial indicators

Project No.: Z01 ES 60012-01 EMB

1. Environmental Mutagenesis Branch
2. Somatic Cell Genetics Section
3. Research Triangle Park, NC

PHS-NIH

Individual Project Report

July 1, 1974 through June 30, 1975

PROJECT TITLE: "Interaction of Heavy Metal Ions with Synthetic DNA Polymers"

PREVIOUS SERIAL NUMBER: None

PRINCIPAL INVESTIGATORS: Michael W. Lieberman, M.D., Ph.D. and
David Amacher, Ph.D.

OTHER INVESTIGATORS: Donald Harvan, M.S. and James B. Patterson, Ph.D.

COOPERATING UNITS: None

MAN YEARS

Total:	1.3
Professional:	0.9
Other:	0.4

PROJECT DESCRIPTION

OBJECTIVES: It is now widely appreciated that heavy metals may have deleterious effects on living organisms. Because of the variety of the metals involved and the diversity of the effects, it is likely that a number of different mechanisms are involved. Recent studies have indicated that some heavy metals are mutagenic and they may form physical complexes with DNA and chromatin. To learn more about the interaction of metal ions with DNA, we have begun by studying the effect of Hg^{2+} and Ag^+ on the buoyant density of synthetic DNA polymers of defined sequence. It is hoped that eventually such studies will be useful in elucidating the mechanism by which genetic alterations occur.

METHODS EMPLOYED: Analytical and preparative density equilibrium centrifugation in Cs_2SO_4 ; spectrophotometric thermal denaturation studies; thin-layer chromatography.

MAJOR FINDINGS: When Hg^{2+} or Ag^+ are complexed with synthetic DNA polymers of different base sequence, a variety of interacting changes are noted: Hg^{2+} results in a large increase in the buoyant density of poly (dA-dT), but has little effect on the densities of poly(dA).poly(dT), poly(dG-dC), or poly (dG).poly(dC). Hg^{2+} causes strand separation of poly(dA).poly(dT), probably by binding extensively to intrastrand thymines. Ag^+ , on the other hand, induces a large change in the buoyant density of poly(dG-dC) and has a lesser

effect on other polymers. These results imply a selectivity of binding of these metal ions to DNA sequences which is based, at least in part, on nearest neighbor sequence.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: These findings have a number of implications for the types of genes which are mutable by metal ions: Ag^+ , for instance, attacks poly(dG-dC) sequences much more readily than poly(dG)-poly(dC). As a consequence, it may be more mutagenic for sequences rich in GCGCGC stretches than for ones rich in GGGGG sequences. In addition, these findings allow us to explain much of the heretofore unexplained behavior of naturally occurring DNA sequences (satellite DNAs, 5s DNA, rDNA) in metal ion - Cs_2SO_4 gradients and to devise methodology for isolation of specific genes.

PROPOSED COURSE: We propose to expand our studies to look at Au, Ni and Pt ions.

KEYWORD DESCRIPTORS: Heavy metals, DNA polymers, DNA binding

Project No.: Z01 ES 60013-01 EMB
1. Environmental Mutagenesis Branch
2. Somatic Cell Genetics Section
3. Research Triangle Park, NC

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

PROJECT TITLE: "Interaction of Mutagens and Carcinogens with DNA and Polydeoxyribonucleotides"

PREVIOUS SERIAL NUMBER: None

PRINCIPAL INVESTIGATORS: Donald P. Harvan, M.S. and Michael W. Lieberman, M.D., Ph.D.

OTHER INVESTIGATORS: Ronald Hass, Ph.D.

COOPERATING UNITS: Chemistry Section, EBCB, NIEHS

MAN YEARS

Total: 0.4
Professional: 0.2
Other: 0.2

PROJECT DESCRIPTION

OBJECTIVES: One of the most exciting developments in the field of chemical carcinogenesis is the observation that many chemical carcinogens are mutagens. Although previous studies have demonstrated that direct-acting carcinogens bind covalently to DNA, relatively little work has been done on the type of adducts formed and especially on the role of base sequence and nearest neighbors on binding. Such information would be of great use in the understanding of the mutagenic effects of carcinogens and in helping to identify regions of the genome which might be hypersensitive to chemical attack. We have begun such studies by looking at the binding of a model carcinogen (N-acetoxy-2-acetylaminofluorene) to four synthetic polymers: poly (dA-dT); poly (dA) · poly (dT); poly (dG-dC) and poly (dG) · poly (dC).

METHODS EMPLOYED: Enzymatic digestion studies; thin-layer and column chromatography; mass spectroscopy.

MAJOR FINDINGS: At present we are still developing methodology. To date our efforts have been directed towards the study of the interaction of 9-¹⁴C-N-acetoxy-2-acetylaminofluorene and four polydeoxyribonucleotides: poly(dA-dT); poly(dA)·poly(dT); poly(dG-dC); poly(dG)·poly(dC).

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Definition of the products of reaction between carcinogens and/or mutagens and DNA

is a prerequisite for any comprehensive understanding of the molecular basis of chemical mutagenesis.

PROPOSED COURSE: To carry out the above outlined research and eventually begin to examine other selected chemical mutagens and naturally occurring DNAs of defined sequence.

KEYWORD DESCRIPTORS: DNA binding, polydeoxyribonucleotide, mutagens, carcinogens.

Project No.: Z01 ES 60014-01 EMB
1. Environmental Mutagenesis Branch
2. Somatic Cell Genetics Section
3. Research Triangle Park, NC

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

PROJECT TITLE: "Removal of Bound Carcinogen from the DNA of Cultured Mammalian Cells during Repair Synthesis"

PREVIOUS SERIAL NUMBER: None

PRINCIPAL INVESTIGATOR: David E. Amacher, Ph.D. and John A. Elliott, Ph.D.

OTHER INVESTIGATOR: Michael W. Lieberman, M.D., Ph.D.

COOPERATING UNITS: None

MAN YEARS

Total:	1.2
Professional:	1.2
Other:	0.0

PROJECT DESCRIPTION

OBJECTIVES: A variety of mammalian cells initiate DNA repair synthesis in response to damage by chemical mutagens and carcinogens. Usually this process is studied by following the incorporation of radioactive precursors into non-replicating DNA. Much less work has been done on the removal of bound carcinogen; however, analysis of the removal of covalently bound mutagens and carcinogens is of great importance since this approach allows quantitation of the amount of damage actually removed during repair. To help assess the importance of repair synthesis in mutagenesis and carcinogenesis, we have begun to examine the removal of bound acetylaminofluorene (a potent chemical carcinogen and mutagen) and thymine dimers produced by ultraviolet radiation from a number of mammalian cell lines. Such studies should allow quantitative estimation of the extent of removal bound carcinogen and rates of removal of different adducts in different cell lines.

METHODS EMPLOYED: Isotopic-labeling, CsCl isopycnic centrifugation, cell culture, thin-layer chromatography.

MAJOR FINDINGS: All cell types examined thus far, including mouse cells (Balb/c, 3T3), guinea pig embryo cells, rat kangaroo cells and human diploid fibroblasts (WI-38), remove substantial bound acetylaminofluorene (30-60%) in 24 hours. Surprisingly, mouse cells which are supposedly less repair-competent than human cells remove more bound carcinogen than human cells. At present we are comparing the rates of removal of bound carcinogen and thymine dimers in the different cell lines.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: It is hoped that these studies will provide a clearer understanding of the role of repair in modifying mutagenesis and perhaps carcinogenesis. Our initial studies indicate that assessment of repair competence on the basis of thymine dimer removal alone may be misleading, especially for evaluation of processes related to mutagenesis and carcinogenesis.

PROPOSED COURSE: The project is in its early stages and will be continued along the same lines.

KEYWORD DESCRIPTORS: Repair synthesis, cultured mammalian cells, carcinogen

Project No.: Z01 ES 60015-01 EMB
1. Environmental Mutagenesis Branch
2. Somatic Cell Genetics Section
3. Research Triangle Park, NC

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

PROJECT TITLE: "Analysis of the Effects of Mutagens and Carcinogens on
Nuclear Protein Synthesis in Cultured Human Diploid Cells"

PREVIOUS SERIAL NUMBER: None

PRINCIPAL INVESTIGATOR: Michael W. Lieberman, M.D., Ph.D. and Gary S. Stein, Ph.D.

OTHER INVESTIGATOR: None

COOPERATING UNIT: Department of Biochemistry, University of Florida, School
of Medicine, Gainesville, Florida

MAN YEARS

Total: 0.5

Professional: 0.2

Other: 0.3

PROJECT DESCRIPTION

OBJECTIVES: Cultured human cells repair DNA damage induced by ultraviolet radiation and chemical mutagens and carcinogens. Little is known, however, about the fate of DNA-associated proteins (histones and non-histone nuclear proteins) during repair synthesis. An understanding of the metabolism of these proteins is of importance since rearrangements or derangements of their structure or function could lead to alterations in gene expression or activation with important cellular consequences. We have begun an investigation in this area by examining the rates of synthesis of histones and acidic nuclear proteins during repair synthesis.

METHODS EMPLOYED: Cell culture; isotopic labeling; CsCl equilibrium centrifugation; gel electrophoresis; liquid scintillation counting.

MAJOR FINDINGS: Human cells repair damage to DNA induced by ultraviolet radiation and N-acetoxy-2-acetylaminofluorene (NA-AAF) without the synthesis of new histones or acidic nuclear proteins.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Since about 100 bases (10 turns of the double helix) are removed during repair of NA-AAF or UV damage, the above result suggests either that damage occurs primarily in stretches not covered by nuclear proteins or (more likely) that nuclear proteins are reutilized (conserved) during repair synthesis.

Alternatively, regions in which repair has occurred remain naked. Distinguishing among these models is of great importance since it may allow one to examine how mutagenesis and gene expression interact.

PROPOSED COURSE: We propose to devise experiments to distinguish among these possibilities, to analyze the distribution of damage induced by these agents and to begin studies of nuclear protein alteration (phosphorylation, acetylation, methylations, etc.).

KEYWORD DESCRIPTORS: Mutagen, carcinogen, nuclear protein, repair synthesis

Project No.: Z01 ES 60016-01 EMB

1. Environmental Mutagenesis Branch
2. Somatic Cell Genetics Section
3. Research Triangle Park, N. C.

PHS-NIH

Individual Project Report

July 1, 1974 through June 30, 1975

PROJECT TITLE: "In vitro Transformation and Mutagenesis in Cultured Mammalian Cells"

PREVIOUS SERIAL NUMBER: None

PRINCIPAL INVESTIGATORS: John A. Elliott, Ph.D., Shiu L. Huang, Ph.D.,
Yulaka Ishii, Ph.D.

OTHER INVESTIGATOR: Michael W. Lieberman, M. D., Ph.D.

COOPERATING UNITS: None

MAN YEARS

Total:	0.7
Professional:	0.7
Other:	0.0

PROJECT DESCRIPTION

OBJECTIVES: Many recent studies have demonstrated that carcinogens act as mutagens in microbial test systems and cultured mammalian cells. It is unclear, however, whether the mutagenic action of carcinogenesis is related to their oncogenic potential or is an unrelated phenomenon. Since carcinogens have a wide variety of other effects on living cells including alterations in DNA, RNA and protein synthesis, membrane changes, changes in gene expression, and toxic effects, it would be of interest to begin to evaluate the role of these events and mutagenesis in the oncogenic process. We have begun to evaluate the role of these events and mutagenesis in the oncogenic process. We have begun to examine mutagenesis and in vitro transformation simultaneously in the same cell lines and to evaluate the dosimetry of these processes using isotopically labeled carcinogens. The objective is to help clarify what role, if any, mutagenesis plays in transformation and carcinogenesis.

METHODS EMPLOYED: Cell culture, mutagenesis assay (HGPRT locus), transformation assay (growth in soft agar), radioisotope techniques, CsCl isopycnic centrifugation, thin-layer chromatography.

MAJOR FINDINGS: The project has only recently begun. Since mutation assays and transformation assays take 4-6 weeks in mammalian cells, we have no data from this project.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE:

Hopefully this project should help clarify the relationship between mutagenesis and carcinogenesis. At present no data are available.

PROPOSED COURSE: Proceed as indicated above.

KEYWORD DESCRIPTORS: Transformation, mutation, mammalian cell culture, carcinogen, mutagen.

Project No.: Z01 ES 60017-01 EMB

1. Environmental Mutagenesis Branch
2. Somatic Cell Genetics Section
3. Research Triangle Park, N. C.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

PROJECT TITLE: "Measurement of Spontaneous and Induced Mutant Frequencies in Human Diploid Fibroblasts"

PREVIOUS SERIAL NUMBER: None

PRINCIPAL INVESTIGATOR: Shiu L. Huang, Ph.D.

OTHER INVESTIGATOR: Michael W. Lieberman, M. D., Ph.D.

COOPERATING UNITS: None

MAN YEARS

Total:	0.3
Professional:	0.3
Other:	0.0

PROJECT DESCRIPTION

OBJECTIVES: Although a substantial body of knowledge exists on the mutagenic effects of chemicals and radiation in prokaryotes and lower eukaryotes, little is known about the mechanism of mutagenesis in human diploid cells. Clearly, if microbial test systems are to be used to evaluate the carcinogenic and mutagenic potential of environmental chemicals, knowledge of how microbial systems relate to human cells is essential. We have, therefore, begun a large project to investigate the induced forward mutation frequency at a haploid locus (X linked locus, HGPRT) and at a diploid locus (autosomal locus, AGPRT) in human diploid fibroblasts. In addition, the induced revertant mutant frequency (HGPRT in Lesch Nyan cells) in human fibroblasts in a variety of sibships will be investigated. Partial characterization of these mutations will be possible with the use of selected compounds (ICR-170, ICR-191; MNNG; NA-AAF). We will also do the dosimetry of induced mutation frequency using radiolabeled MNNG and NA-AAF.

METHODS EMPLOYED: Cell culture, mutagenesis assays, applied enzymology, radioisotope techniques, CsCl centrifugation.

MAJOR FINDINGS: None; our first experiments are still in progress.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: It is hoped that this study will broaden our understanding of mutagenesis in human

cells and allow more direct application of the findings in microbial test systems to man.

PROPOSED COURSE: To investigate the induced forward mutation frequency at a haploid locus (X linked locus, HGPRT) and at a diploid locus (autosomal locus, AGPRT) in human diploid fibroblasts in order to gain understanding of how microbial systems relate to human cells. Additionally, the induced revertant mutant frequency (HGPRT in Lesch Nyan cells) in human fibroblasts in a variety of sibships will be investigated. Partial characterization of these mutations will be possible with the use of selected compounds (ICR-170, ICR-191; MNNG; NA-AAF). Dosimetry of induced mutation frequency using radiolabeled MNNG and NA-AAF also will be done.

KEYWORD DESCRIPTORS: Mutagenesis, human diploid cells, HGPRT, AGPRT.

Project No.: Z01 ES 60018-01 EMB

1. Environmental Mutagenesis Branch
2. Somatic Cell Genetics Section
3. Research Triangle Park, N. C.

PHS-NIH

Individual Project Report

July 1, 1974 through June 30, 1975

PROJECT TITLE: "Isolation of Human Ribosomal Cistrons"

PREVIOUS SERIAL NUMBER: None

PRINCIPAL INVESTIGATORS: Michael W. Lieberman, M.D., Ph.D., and Darrel
Stafford, Ph.D.*

OTHER INVESTIGATORS: None

COOPERATING UNIT: *Department of Zoology, University of North Carolina, Chapel
Hill, N. C. 27514

MAN YEARS

Total:	0.3
Professional:	0.3
Other:	0.0

PROJECT DESCRIPTION

OBJECTIVES: It is clear that mutation cannot be understood in molecular terms without a detailed knowledge of the base sequence and structure of the genes involved. A prerequisite for this type of analysis is physical isolation of the genes of interest. Our initial effort has been directed toward the isolation of the human ribosomal cistrons. (the genes which code for ribosomal RNA). Isolation and characterization of these cistrons should provide a substrate for analyzing the interaction of these genes with chemical mutagens and carcinogens and allow a detailed map of "hot spots" within these sequences. In addition, the experience will provide approaches for isolating other genes from human DNA.

METHODS EMPLOYED: DNA isolation techniques including enzymatic digestion, phenol extraction, shearing, Hg^{2+} - and Ag^{+} Cs_2SO_4 density gradient centrifugation; Actinomycin D - CsCl density gradient centrifugation; DNA-RNA hybridization.

MAJOR FINDINGS: In one pilot experiment we have been able to prepare DNA from human placenta, shear it to defined size and separate the rDNA genes from the bulk of the DNA in Hg^{2+} - Cs_2SO_4 gradients (see project on Interaction of Heavy Metal Ions . .). This finding combined with selective thermal denaturation techniques developed in Stafford's laboratory should soon allow the isolation of the first human genes.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE:

Analysis of mutagenesis in human cells requires knowledge of the structure of human genes and their susceptibility to attack by mutagens and carcinogens. Any progress towards this goal would be of great importance in our understanding of the molecular events involved in mutation in man.

PROPOSED COURSE: To carry out the isolation of the rDNA sequences from human material and their properties; to investigate the interaction of these sequences with carcinogens and mutagens.

KEYWORD DESCRIPTORS: Ribosomal cistrons, DNA isolation, rDNA.

Project No.: Z01 ES 60019-02 EMB

1. Environmental Mutagenesis Branch
2. Biochemical Genetics Section
3. Research Triangle Park, N. C.

PHS-NIH

Individual Project Report
July 1, 1974 through June 30, 1975

PROJECT TITLE: "The Use of Male Germinal Tissue in the Detection of Mutational Events"

PREVIOUS SERIAL NUMBER: NIEHS-EM-013

PRINCIPAL INVESTIGATOR: Heinrich V. Mallng, Ph.D., and Nivedita Mitra, Ph.D.

OTHER INVESTIGATORS: None

COOPERATING UNITS: None

MAN YEARS

Total:	1.3
Professional:	1.3
Other:	0.0

PROJECT DESCRIPTION

OBJECTIVES: The main aim of this project is to develop systems for measuring the frequency of point mutations in male germinal tissue, i.e., in sperm. Studies along this line involve the use of histochemical staining techniques. The development of such a system would be an advantage because the germ cell samples from man would be readily available for studying the mutation frequency. Besides, it would also provide an assay procedure for determining the effect of chronic levels of exposure because the genetic damage would accumulate in the spermatogonia.

METHODS EMPLOYED: Certain enzymes which occur in sperm can be readily detected by histochemical techniques. The three enzymes used in the present study are: (a) α -glycerophosphate dehydrogenase, (b) lactate dehydrogenase and (c) succinate dehydrogenase.

The principle is that, with the use of standard staining procedures, variation in the co-enzyme or by using different inhibitors specific to the enzyme, it should be feasible to detect individual variant cells.

MAJOR FINDINGS: At present, it has been possible to standardize the staining technique to the extent that it is reproducible after repeated experimentation.

It has also been possible to develop a system of inhibitors specific for each of the enzymes being tested. They are listed below in accordance with the

enzyme.

<u>Enzyme</u>	<u>Inhibitor</u>
1. α -glycerophosphate dehydrogenase	ATP Oxamic acid Malonic acid
2. Lactic dehydrogenase	Urea, Oxamic acid
3. Succinic dehydrogenase	Malonic acid

Experiments are being done with known chemicals to test the validity of the system.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND TO THE PROGRAM OF THE INSTITUTE:

Development of this system is important, because it would permit the detection of mutational events within a single individual that could be used to monitor the human population. Besides, it would also enable us to detect the genetic consequences of exposure to mutagens.

PROPOSED COURSE: The future plans include testing out the present system to determine the spontaneous frequency of variant cells as well as the frequencies induced by various mutagenic agents.

KEYWORD DESCRIPTORS: Germinal tissue, mutation, sperm, enzymes, histochemical.

Project No.: Z01 ES 60020-01 EMB
1. Environmental Mutagenesis Branch
2. Biochemical Genetics Section
3. Research Triangle Park, NC

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

PROJECT TITLE: "Identifying of Chemical Compounds Activated by Germ Cells to Mutagens in Mammals"

PREVIOUS SERIAL NUMBER: None

PRINCIPAL INVESTIGATOR: Peter Maier, Ph.D.

OTHER INVESTIGATOR: Heinrich V. Malling, Ph.D.

COOPERATING UNITS: None

MAN YEARS

Total:	0.4
Professional:	0.4
Other:	0.0

PROJECT DESCRIPTION

OBJECTIVES: Initially this research will attempt to identify compounds which induce point mutations after metabolic activation in the testis of male mice and to develop the protocols necessary for this work. The first series of compounds to be tested will be those which are known to react with testicular tissue. Any compounds that give a high level of mutagenic activation in the testicular microsome test will be tested in a histochemical mutation system using sperm of male mice.

METHODS EMPLOYED: The initial system employed is a testicular microsome system using bacteria as indicator organisms for mutation. Also to be used is a histochemical mutation system in which individual sperm cells are examined for a mutational event.

MAJOR FINDINGS: A manual screening of the literature for compounds which induce cancer of the testis has been undertaken. Most mouse testicular tumors are Leydig cell tumors; many of these, however, are spontaneous in nature. This project was initiated in March 1975.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: It is of importance to future generations to identify compounds that cause point mutations in germinal tissue. A compound which is highly activated by testicular microsomes and induces mutations detected by histochemical methods are likely to induce point mutations in sperm. Such systems as these will aid in preventing unnecessary future genetic hazards.

PROPOSED COURSE: A standardized microsomal test system using mammalian testicular microsomes will be developed. This test system will be used to identify compounds which induce point mutation in germinal tissue. These results will be further substantiated by a histochemical germinal cell mutation system under development.

KEYWORD DESCRIPTORS: Mutation, metabolic activation, testis, mouse, microsomes, bacteria, sperm.

Project No.: Z01 ES 60021-03 EMB
1. Environmental Mutagenesis Branch
2. Biochemical Genetics Section
3. Research Triangle Park, NC

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

PROJECT TITLE: "Electrophoretic Mobility Mutation Analysis in Mammals"

PREVIOUS SERIAL NUMBER: NIEHS-EM-010

PRINCIPAL INVESTIGATOR: Lawrence R. Valcovic, Ph.D.

OTHER INVESTIGATORS: Heinrich V. Malling, Ph.D.

COOPERATING UNITS: None

MAN YEARS

Total:	3.2
Professional:	1.1
Other:	2.1

PROJECT DESCRIPTION

OBJECTIVES: Classically, the detection of point mutations in mammals has relied upon the induction of visible recessive mutations. These studies have not yet identified the molecular event responsible for producing the mutant phenotype. However, the study of specific enzyme mutations using electrophoresis has the capability of detecting mutations that result in a non-functional protein or a functional protein with altered electrical charge. Our objective is to develop a single mammalian test system that will detect both electrophoretic mobility (EPM) and inactive enzyme (IAE) mutations in the same loci. This will enable us to determine the relative frequencies of these two types of mutational events in response to various types of mutagenic agents.

METHODS EMPLOYED: The ratio between mutations resulting in IAE-mutations and EPM-mutations can be estimated from the changes in the zymogram of heterozygous offspring from a cross between two inbred strains which differ in the electrophoretic patterns of several enzymes. In the simplest case let us assume the normal heterozygote will have three bands for a particular enzyme. A mutant induced in either parent can result in one of three different electrophoretic patterns: (a) the presence of any one band indicating that the mutant is inactive; (b) the presence of any two bands, one at the normal position for one of the parents and the other at any position indicating that the inactive enzyme will form an active aggregate with the wild-type enzyme; and (3) the presence of three bands, two with changed positions, indicating an EPM-mutation. Since case (a) and (b) indicate inactive enzymes and (c)

an EPM-enzyme, a direct estimation of the ratio between IAE-mutations and EPM-mutations can be made.

We have selected two inbred mouse strains which differ with respect to nine enzyme loci. Additional non-variant bands will be apparent with some of the strains; and while the number of loci that these represent is unknown, EPM-mutations may be detected at these positions.

Since it is important to correlate this system with Russell's specific locus test in mice, the first mutagen treatment utilized was ionizing radiation. Adult males were irradiated and after a sterile period ranging from 4 to 11 months they were mated to non-irradiated females. The animals received a total of 1000r ^{60}Co gamma rays delivered as two 500r exposures with a dose rate of 90r/min. separated by 24 hours.

Sampling of progeny consists of obtaining blood by orbital puncture and one kidney by surgery. Subsequently, electrophoresis is carried out on the red cell lysate and kidney homogenate from each animal. It is anticipated that approximately 3000 offspring from each of the crosses (DBA x C57; C57 x DBA) will be analyzed for mutations at each of the nine loci showing electrophoretic polymorphism.

MAJOR FINDINGS: Testing for mutations in offspring from DBA/2J treated males mated to C57BL/6J untreated females has progressed. At this writing approximately 1600 animals (14,400 loci) have been examined. Four apparent mutations at different loci have been detected. This tentative mutation rate of 2.8×10^{-4} mutations per locus per gamete is not dissimilar to that obtained in the morphological specific locus test.

Additional treatment of C57BL/6 males at lower radiation doses was completed. Exposure to 700r total dose (350r + 350r) also resulted in extremely high sterility. Further radiation will not be done due to the amount of data being generated from the DBA/2J treated group.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The widespread use of chemicals may increase the genetic load of the human population which may possibly present a health hazard. There are two approaches that may be considered to avoid such an event. First, use animal test systems to detect chemicals which are mutagenic and prohibit or limit their use. Second, use mutant alleles at selected loci in the human population and monitor these for several years to determine whether there is an increase in the frequency of these alleles. The system under development will be pertinent to both of these approaches.

It will survey a large number of different loci to minimize the cost of testing chemicals for potential mutagenicity; and since many of the enzymes are also found in the human population, it will also provide a base-line for the study of the mutation rate of electrophoretic mobility mutations in the human population. Furthermore, since most of the loci code for specific enzymes, purification and biochemical analyses can be employed to determine the nature of the mutational event, i.e., base-pair substitution and/or addition/deletion in the DNA.

PROPOSED COURSE: The radiation experiment will be completed in FY76. Breeding of mutants to obtain homozygotes for biochemical analysis will continue. Procedures for exposing animals to chemical mutagens are being developed and treatment of males of both strains is planned. Efforts to increase the sensitivity of the system by increasing the number of test loci will continue.

KEYWORD DESCRIPTORS: point mutations, mammals, electrophoresis, isozyme, mutagenesis, mutant frequency, monitoring, spermatogonia, radiation.

Project No.: Z01 ES 60022-01 EMB
1. Environmental Mutagenesis Branch
2. Biochemical Genetics Section
3. Research Triangle Park, N. C.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

PROJECT TITLE: "The Heat Stability of Enzymes: An Approach to Measuring Point Mutations in the Mouse"

PREVIOUS SERIAL NUMBER: None

PRINCIPAL INVESTIGATOR: Jean-Claude Mareschal, Ph.D.

OTHER INVESTIGATORS: Lawrence R. Valcovic, Ph.D., and Heinrich V. Mallng, Ph.D.

COOPERATING UNITS: None

MAN YEARS

Total:	0.9
Professional:	0.7
Other:	0.2

PROJECT DESCRIPTION

OBJECTIVES: Point mutations within inbred strains of mice will be studied using some biochemical properties and/or the heat stability properties of enzymes. These studies will provide an increased number of loci to be examined in mouse biochemical specific locus tests.

METHODS EMPLOYED: After incubating the mouse kidney homogenate of different mouse strains at given temperatures for a specific time period, the enzymes were assayed spectrophotometrically. Curves were plotted for each set of conditions and each enzyme to determine any heat stability differences. Samples were also examined using electrophoresis.

MAJOR FINDINGS: We have found that there are important differences in the heat stability curves of several enzymes from DBA/2J and C57BL/2J strains of mice. These enzymes are glucose-6-phosphate dehydrogenase, glucose-6-phosphate isomerase, isocitrate dehydrogenase, phosphoglycerate kinase, dipeptidases and esterases. This project was initiated in January 1975.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Heat-stability studies will expand the number of loci that can be studied in the mouse biochemical specific locus mutation system. This will allow the assay to be more sensitive, less expensive and less time consuming. These studies will also aid mapping of new loci in the mouse genome.

PROPOSED COURSE: Our study will be extended to a minimum of twenty enzymes having differences in heat stability in DBA/2J and C57BL/6J strains of mice. The next step will be to look for the behavior of these enzymes in offspring after the parents have been treated with mutagens.

KEYWORD DESCRIPTORS: mutation, heat-stability, enzymes, mouse, electrophoresis, kidney, specific locus.

Project No.: Z01 ES 60023-01 EMB

1. Environmental Mutagenesis Branch
2. Biochemical Genetics Section
3. Research Triangle Park, NC

PHS-NIH

Individual Project Report

July 1, 1974 through June 30, 1975

PROJECT TITLE: "The Comparison and Evaluation of Mutagenic Test Systems for Mass Screening Systems"

PREVIOUS SERIAL NUMBER: None

PRINCIPAL INVESTIGATORS: Larry D. Claxton and Heinrich V. Malling, Ph.D.

OTHER INVESTIGATORS: None

COOPERATING UNITS: None

MAN YEARS

Total:	1.3
Professional:	0.3
Other:	1.0

PROJECT DESCRIPTION

OBJECTIVES: The purpose of this study is to evaluate and compare the usefulness, reliability and scientific soundness of presently available mutation test systems using a variety of mutagenic agents. This project will attempt to provide standardized protocols and computerized control of tests that can be used in future hierarchal test systems commonly called Tier tests. This evaluation would aid in the comparison of results from several test systems and in the extrapolation to human studies. The computer programming of test systems would insure objective analysis and comparison of data which is not presently available.

METHODS EMPLOYED: Several known mutagens that have different mechanisms of genetic action and different physical and chemical characteristics are used in each of the selected test systems. The parameters of each system are varied in such a manner as to optimize the system for screening conditions. A comparison is then made between the sensitivity, reliability, etc., of the various systems used. Included within the systems being tested are several bacterial systems, activation systems, a cell culture system and a host-mediated assay system.

MAJOR FINDINGS: Although this work has just begun, preliminary results with known mutagens in these test systems do give positive results. The computer programming of the microsomal bacterial test system is now in its early stages and appears feasible.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: This evaluation and comparison will provide scientifically sound basis for large-scale screening and testing of environmental chemicals for mutagenicity. This study will also provide answers as to the feasibility of extrapolating results from one system to another.

PROPOSED COURSE: Future efforts of this project will include the standardizing of the test systems now being evaluated and the determining of proper controls for mass screening. The computerized programming of the microsomal assay system will also be completed.

KEYWORD DESCRIPTORS: Mutation, mass screening, bacterial systems, activation, cell culture, host-mediated assay, environmental chemicals, computer.

Project No.: Z01 ES 60024-01 EMB

1. Environmental Mutagenesis Branch
2. Mammalian Genetics & Cytogenetics Section
3. Research Triangle Park, NC

PHS-NIH

Individual Project Report

July 1, 1974 through June 30, 1975

PROJECT TITLE: "Effects of Dietary Deficiency on Sensitivity to Mutation Induction"

PREVIOUS SERIAL NUMBER: None

PRINCIPAL INVESTIGATOR: William Sheridan, Ph.D.

OTHER INVESTIGATORS: E. R. Soares, Ph.D. and Ruth M. Sorg, M.S.

COOPERATING UNITS: None

MAN YEARS

Total:	1.3
Professional:	0.7
Other:	0.6

PROJECT DESCRIPTION

OBJECTIVES: In irradiation studies with mice, it has been found that diets deficient in one or more of the vitamins or trace minerals lead to an increase of the somatic sensitivity of the animal to induction of damage. Somatic and genetic sensitivity have been shown to be correlated in the mouse. It is our purpose to investigate whether or not dietary deficiency might change the overall state of the animal making it more susceptible to the actions of mutagenic agents.

METHODS EMPLOYED: Inbred mice of the C57BL/6 strain were placed on a diet deficient in Vitamin D and after an appropriate adaptation time were mated in single pair matings for 100 days. F₁ offspring were continued on the diet until maturity. Male offspring were treated with a known chemical mutagen (TEM) and outcrossed in a dominant lethal test. Male offspring were also treated with the agent and bred to CD₁ females to produce offspring for cytogenetic analysis of the induction of transmissible translocations.

MAJOR FINDINGS: There were no overt indications that the deficient diet produced morphological changes in the offspring or caused untoward effects on the reproductive capacity of the mice subjected to it. Preliminary findings in the dominant lethal test suggest that there may be an increase in foetal mortality among offspring to TEM treated males reared on the diet as compared to TEM treated males given a full diet.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: A greater part of the human population on a world-wide basis is known to be subjected to nutritional insufficiency at the present time. At the same time it is known that there are present in the human environment compounds which are mutagenic. Knowledge of the existence of effects of dietary deficiency on sensitivity to mutation induction will be a necessary consideration in attempts to extrapolate from information derived from laboratory animals in estimating risks to the human population.

PROPOSED COURSE: The cytogenetic analysis will be made when offspring to treated animals reach maturity. Other dietary deficiencies will be investigated to determine if there are specific deficiencies which change sensitivity to mutagenic compounds or if the effects are general in nature.

KEYWORD DESCRIPTORS: Dietary deficiency, mice, cytogenetic analysis, dominant lethal, translocations, chemical mutagen, genetic sensitivity.

Project No.: Z01 ES 60025-01 EMB

1. Environmental Mutagenesis Branch
2. Mammalian Genetics & Cytogenetics Section
3. Research Triangle Park, NC

PHS-NIH

Individual Project Report

July 1, 1974 through June 30, 1975

PROJECT TITLE: "A Study of the Dominant Lethal Effects of AF-2"

PREVIOUS SERIAL NUMBER: None

PRINCIPAL INVESTIGATOR: Eugene R. Soares, Ph.D.

OTHER INVESTIGATOR: William Sheridan, Ph.D.

COOPERATING UNITS: None

MAN YEARS

Total:	0.9
Professional:	0.3
Other:	0.6

PROJECT DESCRIPTION

OBJECTIVES: This investigation was designed to study the possible effects (e.g., toxicity and induced dominant lethals) of the food additive AF-2 in mice.

METHODS EMPLOYED: Initially, several toxicity tests were conducted employing male mice of three different inbred strains and using several doses of AF-2. All mice were injected by gavage and were observed for any toxic effects of the compound. Following these tests, 150 strain DBA/2J males were randomized into 5 groups of 30 mice each, treated with AF-2 at doses ranging from 0.0 mg/kg to 450 mg/kg; and then tested according to the dominant lethal assay.

MAJOR FINDINGS: AF-2 in the strains tested was relatively non-toxic at doses of up to 900 mg/kg of body weight. However, in those tests where the mice were deliberately starved of food and water for 24 hours prior to treatment, significant increases in AF-2 related deaths were noted.

Results obtained in the dominant lethal test showed no indication of AF-2 induced dominant lethality. We observed no AF-2 related increases in fetal mortality nor any decreases in live or total implants. It was also apparent from this study that AF-2 had no effect on male fertility.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: AF-2 is a known carcinogen in mice. An important aspect of mutagen testing is the elucidation of the relationship between carcinogenicity and mutagenicity.

This work was designed to further clarify in a mammalian system the above relationship.

PROPOSED COURSE: This project was completed in January 1975.

KEYWORD DESCRIPTORS: Food additive, mice inbred strains, gavage, dominant lethal, AF-2, starved, carcinogen, carcinogenicity, mutagenicity, mammalian.

PUBLICATIONS

E. R. Soares and W. Sheridan (1975) Lack of Induction of Dominant Lethals in Mice by Orally Administered AF-2. Mutation Research (in press).

Project No.: Z01 ES 60026-02 EMB

1. Environmental Mutagenesis Branch
2. Mammalian Genetics & Cytogenetics Section
3. Research Triangle Park, NC

PHS-NIH

Individual Project Report

July 1, 1974 through June 30, 1975

PROJECT TITLE: "Studies of the Effects of Route of Administration of the Sensitivity of Different Mouse Strains to Mutagenic Agents"

PREVIOUS SERIAL NUMBER: NIEHS-EM-018

PRINCIPAL INVESTIGATOR: Eugene R. Soares, Ph.D.

OTHER INVESTIGATOR: William Sheridan, Ph.D.

COOPERATING UNITS: None

MAN YEARS

Total:	1.0
Professional:	0.6
Other:	0.4

PROJECT DESCRIPTION

OBJECTIVES: Earlier studies designed to test the toxicological sensitivity of various compounds have shown marked differences in the sensitivity between the various strains used. Our present studies use known chemical mutagens and are designed to confirm the above finding as well as determine variability of mutagenic sensitivity between routes of administration and between and within strains of mice.

METHODS EMPLOYED: Mice of two inbred strains and one hybrid are each given three different dose levels of ethyl methanesulfonate. Similarly male mice of one inbred strain are given an injection of triethylenemelamine. In both studies the chemicals are administered by one of two routes, either intraperitoneally (i.p.) or by gavage. All mice are observed for the toxic effects of the compounds and subsequently mated according to the standard scheme employed in the dominant lethal assay.

MAJOR FINDINGS: In the studies using EMS, marked differences in toxicological sensitivity between strains were noted. Furthermore, i.p. administration of EMS was more effective than oral administration. No differences between or within strains were found with respect to EMS-induced dominant lethality.

Although, TEM, at the dose used in this study, was relatively non-toxic to the mice regardless of the route of administration, the rate of induction of dominant lethality was clearly route dependent. When injected i.p., TEM induced significant increases in fetal mortality during the first three weeks following treatment. In contrast oral administration of TEM resulted in a

moderate, but not statistically significant, increase in fetal mortality in week one only with no apparent effect in weeks two and three.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Test systems, as they are currently designed, are restricted to the use of one or a few inbred strains and, with few exceptions, consider only the effects of i.p. injections of the test compounds. Since human populations are genetically heterogeneous and since exposure of humans to various compounds, either toxic or mutagenic, is by routes other than i.p., it is extremely important that the varying effects of genetic background and route of administration be tested. Such effects must be studied before reliable screening procedures can be developed which would allow extrapolation to human populations or estimates of risk.

PROPOSED COURSE: Future efforts will be directed toward elucidating the varying effects of a series of different compounds on a number of strains. Efforts will also be made to determine the cause for the different patterns of observed route dependent sensitivities.

KEYWORD DESCRIPTORS: Route of administration, strains, mutagenic agents, toxicological sensitivity, mice, ethyl methanesulfonate, triethylenemelamine, i.p., gavage, dominant lethal assay.

PUBLICATIONS

Soares, E. R. and Sheridan, W. (1975) Route of administration dependent differences in TEM-induced dominant lethals. Environmental Mutagen Society Annual Meeting, Miami, Florida. (Abstract)

Project No.: Z01 ES 60027-02 EMB

1. Environmental Mutagenesis Branch
2. Mammalian Genetics & Cytogenetics Section
3. Research Triangle Park, NC

PHS-NIH

Individual Project Report

July 1, 1974 through June 30, 1975

PROJECT TITLE: "Comparisons of the Effects of Chemical Mutagens on Somatic Cells and Germinal Cells in Mice"

PREVIOUS SERIAL NUMBER: NIEHS-EM-017

PRINCIPAL INVESTIGATOR: Eugene R. Soares, Ph.D.

OTHER INVESTIGATORS: Ruth M. Sorg, M.S.

COOPERATING UNITS: None

MAN YEARS

Total:	0.6
Professional:	0.2
Other:	0.4

PROJECT DESCRIPTION

OBJECTIVES: This project will be designed to explore the feasibility of using somatic cells to screen for chemically induced chromosomal abnormalities. Inclusive in this project is the study of the relative sensitivities to chemical mutagens of germ cells (sperm) and lymphocytes.

METHODS EMPLOYED: Adult male mice will be exposed to known chemical mutagens. Subsequent to exposure blood will be withdrawn from these animals, cultured, and the lymphocytes prepared for cytological examination and karyotyping. Also, following treatment, males will be mated according to the procedure used in the dominant lethal assay. Testicular sections from treated males as well as sperm from the vas and epididymis will be examined for chromosome abnormalities and sperm head abnormalities, respectively.

It should be noted that in an earlier description of this project it was proposed that female germ cells as well as zygotes be exposed to chemical mutagens; such treatment to be followed by appropriate cytological examinations. This portion of the project has since been revised and modified and will be described elsewhere.

MAJOR FINDINGS: Considerable difficulty was encountered regarding technical problems involved in the culturing of mouse lymphocytes.

Many of the technical problems (i.e., strain differences in transformation; sufficient numbers of countable metaphase plates) have been resolved and work will be continued along the line described above.

Studies comparing the effects of chemicals as measured by the dominant lethal assay and the sperm morphology technique are still in progress. Preliminary data available from these studies have not shown any clear cut correlation between the rate of induction of lethals and increased percentages of abnormal sperm.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: A major drawback in using mammals for screening for chemical mutagens is the relatively long generation time as compared to lower organisms. The use of somatic cells for screening procedures would eliminate this problem and would allow rapid and reliable screening of large numbers of animals. This project will provide information concerning the relative sensitivities of these cells and may enable extrapolation from somatic cells to germinal cells. Similarly, such information could easily be adapted to use among human populations.

PROPOSED COURSE: A variety of chemical agents will be tested in several strains and hybrid mice. The effects of acute and chronic administration of the compounds will be considered.

KEYWORD DESCRIPTORS: Chemical mutagens, somatic cells, germinal cells, mice, lymphocytes, sperm, sperm head abnormalities, dominant lethal assay.

Project No.: Z01 ES 60028-01 EMB

1. Environmental Mutagenesis Branch
2. Mammalian Genetics & Cytogenetics Section
3. Research Triangle Park, NC

PHS-NIH

Individual Project Report

July 1, 1974 through June 30, 1975

PROJECT TITLE: "Genetic Effects on Early Mouse Embryos After Mutagenic Treatment of the Parents"

PREVIOUS SERIAL NUMBER: None

PRINCIPAL INVESTIGATORS: Kurt Burki, Ph.D. and William Sheridan, Ph.D.

OTHER INVESTIGATORS: None

COOPERATING UNITS: None

MAN YEARS

Total:	0.7
Professional:	0.7
Other:	0.0

PROJECT DESCRIPTION

OBJECTIVES: One of the most widely used tests of mutagenicity in mammals is the dominant lethal assay. While this system allows detection of induced increases in foetal mortality, the underlying causes are not clearly delineated. One of the proposed factors has been the induction of chromosomal aberrations. It is our purpose to investigate the processes and causes of foetal mortality by direct observation of the development of embryos and by cytogenetic analysis of their karyotypes.

METHODS EMPLOYED: The methodology of culturing mouse embryos in vitro is being developed and perfected. When constantly satisfactory culturing is achieved, male and/or female mice will be treated with a known chemical mutagen. The treated animal will then be mated to untreated animals to allow in vivo fertilization. Twenty-four hours post mating the embryos will be retrieved from the oviducts of the females and established in cultures. Approximately 48 hours post culture, at the 8-16 cell stage, embryos will be prepared for cytological examination and the rate of induction of chromosomal abnormalities determined.

MAJOR FINDINGS: Project commenced February 1975.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Chromosome aberrations in man are known to cause disturbances in development of embryos leading to death of the embryo or to abnormal offspring. The project

described here is designed to develop an understanding of the fate and effects of spontaneous and induced chromosome aberrations at various stages of embryogenesis.

The system when fully developed may permit the detection of possible mutagenic effects of potential mutagens in the human environment.

PROPOSED COURSE: Mouse eggs will be fertilized in vivo or in vitro and the embryos cultured in vitro up to blastocyst stages. Fertilization will be carried out with normal and mutagen treated sperm. Rates of development and rates of chromosome aberrations at various stages of embryogenesis will be determined. Results with in vitro culture will be compared to results from in vivo embryogenesis.

KEYWORD DESCRIPTORS: Mouse, embryos, in vitro culture, cytogenetics, chromosome aberrations, mutagens.

Project No.: Z01 ES 60029-02 EMB

1. Environmental Mutagenesis Branch
2. Population Genetics & Epidemiology Section
3. Research Triangle Park, NC

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

PROJECT TITLE: "Genetic Variability and Its Relationship to Environmental Variation"

PREVIOUS SERIAL NUMBER: NIEHS-EM-025

PRINCIPAL INVESTIGATORS: Charles H. Langley, Ph.D. and John H. Gillespie, Ph.D.

OTHER INVESTIGATORS: Diana Smith and Kenneth S. Crump, Biometry Branch

COOPERATING UNITS: Environmental Biometry Branch

MAN YEARS

Total:	3.0
Professional:	2.5
Other:	0.5

PROJECT DESCRIPTION

OBJECTIVES: Recent studies have implicated environmental polymorphism as an important parameter in the interpretation of existing genetic polymorphism, especially allozymic polymorphism. We are attacking the question from two angles: (1) theoretical modeling of evolution dynamics in the presence of environmental variation and (2) experimental investigations of the relative sensitivity of various genotypes to environmental hazards. In cooperation with statisticians in the Environmental Biometry Branch and geneticists at North Carolina State University, we are analyzing gene frequency changes in response to insults of environmental chemicals. This will give some indication of the responsiveness of gene frequencies to environmental contamination.

METHODS EMPLOYED: In the theoretical analyses, Monte Carlo computer simulations are being run to investigate the relationships of environmental variability to genetic variability. In experimental work, laboratory populations of *Drosophila* are presently being utilized. These are killed off to a large extent (90%) with environmental agents and the gene frequencies at allozyme loci analyzed.

MAJOR FINDINGS: In the theoretical analysis it has been possible to model the behavior of alleles with various gene activity in varying environments. The equilibrium state of multilocus systems in stochastic environments has also been determined for several specific models. In the experimental area we have observed gene frequency change in response to a single generation exposure to environmental extremes.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: In recent years it has become apparent that there is an enormous amount of genetic variability present in natural populations, including humans. The adaptive significance of this variation must be understood in order that we can judge the implication of new environmental changes and the genetic consequences of mutagenic environmental agents.

PROPOSED COURSE: If gene frequency changes prove to be indicators of environmental insult, we plan to investigate natural populations for evidence of response to known environmental contaminations.

KEYWORD DESCRIPTORS: Genetic variability, environmental variation, exposure to environmental extremes.

PUBLICATIONS

Gillespie, J. H. and Langley, C. H.: A general model to account for enzyme variation in natural populations. Genetics 76:837-884, 1974.

Gillespie, J. H. and Langley, C. H.: Multilocus behavior in random environments. I. Random Levene Models (submitted).

Project No: Z01 ES 60030-01 EMB

1. Environmental Mutagenesis Branch
2. Population Genetics & Epidemiology Section
3. Research Triangle Park, N.C.

PHS-NIH

Individual Project Report

July 1, 1974 through June 30, 1975

PROJECT TITLE: "Comparison of Mutation Rates in Natural and Laboratory Environments"

PREVIOUS SERIAL NUMBER: None

PRINCIPAL INVESTIGATOR: Charles H. Langley, Ph.D.

OTHER INVESTIGATORS: Kazuko Ito, Ph.D.

COOPERATING UNITS: None

MAN YEARS

Total:	1.0
Professional:	0.5
Other:	0.5

PROJECT DESCRIPTION

OBJECTIVE: To compare mutation rates in natural and laboratory environments to determine whether the natural population shows a significant increase in the recessive lethal rate.

METHODS EMPLOYED: Sperm samples were obtained from Drosophila melanogaster males in a natural population and similar males reared in the laboratory from wild caught females. Sex-linked recessive lethals were scored from each type of males.

MAJOR FINDINGS: There was no difference in the mutation rates, and the actual difference could not be larger than 0.17 percent. It is important to establish that the laboratory environment is not radically different from that in a natural population. Furthermore, it suggests that the high mutation rates recently observed with natural chromosomes are associated with genetic background sensitivity.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The relevance of this research is simply the validation of control recessive lethal mutation rates in Drosophila.

PROPOSED COURSE: To be continued.

KEYWORD DESCRIPTORS: Mutation rates, natural and laboratory environments, sex-linked recessive lethals.

PUBLICATIONS

Langley, C. H. and Ito, K.: Spontaneous mutability in Drosophila melanogaster: In natural and laboratory environments (submitted).

Project No.: Z01 ES 60031-01 EMB

1. Environmental Mutagenesis Branch
2. Population Genetics & Epidemiology Section
3. Research Triangle Park, NC

PHS-NIH

Individual Project Report

July 1, 1974 through June 30, 1975

PROJECT TITLE: "Mathematical Models for Describing the Effect of Migration upon Gene Frequencies"

PREVIOUS PROJECT NUMBER: None

PRINCIPAL INVESTIGATORS: K. S. Crump, Ph.D. and J. H. Gillespie, Ph.D.

OTHER INVESTIGATORS: None

COOPERATING UNITS: Environmental Biometry Branch

MAN YEARS

Total: 0.6

Professional: 0.5

Other: 0.1

PROJECT DESCRIPTION

OBJECTIVES: (1) To construct probabilistic models for determining how far a unique neutral gene will migrate before it disappears from the population and (2) to determine the geographical distribution of gene frequencies given that the gene is still present in the population.

METHODS EMPLOYED: Branching process models, both discrete and continuous time, were used to describe a population experiencing birth, death and migration. Migration is assumed to take place in one or two dimensions.

MAJOR FINDINGS: Mathematical expressions have been derived for the mean and variance-covariance of the numbers of individuals carrying the particular gene that ever appear at various distances. Expressions have also been derived for the correlation, after a long time has elapsed, between the numbers of individuals carrying the gene that are present in two localities separated by various distances.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: An important component in variance in individual responses to environmental insults may be genetic variation. Through this project we are attempting to answer some basic questions about the role of the geographic distribution of populations and migration upon the establishment and maintenance of genetic variability.

PROPOSED COURSE: To consider the predictions of the models when applied to real populations and to compare these models with those of other investigators.

KEYWORD DESCRIPTORS: Population genetics, migration, branching processes, neutral gene.

Project No.: Z01 ES 60032-01 EMB

1. Environmental Mutagenesis Branch
2. Population Genetics & Epidemiology Section
3. Research Triangle Park, NC

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

PROJECT TITLE: "Low Dose Risk Estimation from Experimental Carcinogenesis"

PREVIOUS PROJECT NUMBER: None

PRINCIPAL INVESTIGATORS: D. G. Hoel, Ph.D., K. S. Crump, Ph.D. and
C. H. Langley, Ph.D.

OTHER INVESTIGATORS: C. A. Barnes (University of North Carolina)

COOPERATING UNITS: Environmental Biometry Branch

MAN YEARS

Total:	1.2
Professional:	1.0
Other:	0.2

PROJECT DESCRIPTION

OBJECTIVES: In order to estimate human risk based on animal carcinogenesis experiments it is currently necessary to extrapolate data from high dose experiments to low doses. The purpose of this investigation is to study the various mathematical and probabilistic methods for extrapolation with particular emphasis on those which attempt to relate mechanistically to the carcinogenic process.

METHODS EMPLOYED: Various possible models of carcinogenesis were analyzed with respect to low dose kinetics. Considerations of mechanism such as hit models and multistage models were compared to so-called log normal response models. The importance of background incidence and induction time were evaluated.

These considerations led to the investigation of optimal experimental design considerations. Specifically, the sample size and dose rate were optimized.

MAJOR FINDINGS: (1) If carcinogenesis by an external agent is considered to be augmenting an already ongoing spontaneous process, then the response will be linear at low dose. (2) If the origin of a cancer is an event in a single cell (as distinct from a whole tissue) then the low dose response will be linear. (3) In general, time of cancer induction must be heavily dependent on dose rate for such consideration to play a role in low dose risk. (4) Linear extrapolation is not always the conservative technique it is thought to be.

(5) The optimal experimental dose for obtaining the maximal acceptable dose is robust with respect to models when one is extrapolating linearly. (6) Little is gained by large sample sizes in experiments aimed at low dose risk estimation.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: In order to determine risk associated with possible or actual human exposure of carcinogens it is usually necessary to determine the carcinogenicity of the agent in experimental laboratory animals. The observed risk is then extrapolated down to human exposure levels and then converted to human risk by some conversion factor. The particular model chosen to extrapolate the experimental risk to the low dose risk is critical in determining the order of magnitude of the exposure. This research seeks to investigate the various aspects of this procedure.

PROPOSED COURSE: (1) To modify the hit models to allow a hit cell to be repaired by some stochastic mechanism and determine if repair can have any significant qualitative effect upon the dose response curve. (2) To consider the implication of variance among individuals in sensitivities to an insult of a carcinogen upon low dose risk estimation.

KEYWORD DESCRIPTORS: Carcinogenesis, low dose, extrapolation, risk estimation, models.

ENVIRONMENTAL TOXICOLOGY BRANCH

ENVIRONMENTAL TOXICOLOGY BRANCH

Summary Statement

The scientific efforts of this Branch are directed toward the understanding of mechanisms of toxicity. Special emphasis is placed on environmental problems relating to reproduction and development. These basic studies contribute to the development of better and more effective test procedures to predict toxicity in humans. Available scientific expertise is broad and includes biochemistry, reproductive physiology, pharmacology, and pathology. Test systems are likewise diverse and range from subcellular biochemistry and electron microscopy through in vitro cell and organ cultures to whole animals. Attempts are made to encourage both team and individual scientific efforts. Research activities are organized into three interrelated areas: Experimental Teratology (Dr. Staples), Developmental Toxicology (Dr. Lucier) and Biochemical Toxicology (Dr. Woods).

The Experimental Teratology group is concerned with the etiology of birth defects in mammals, the development of improved test methods for assessing the human teratogenic potential of environmental agents, and the delineation of how teratogens alter the course of development.

Drs. Staples and Maurer are studying the effects of test agents administered directly to the conceptus, rather than to the dam, as a possible means of minimizing differences in toxic responses among laboratory species. Qualitative and quantitative comparisons of responses by the conceptus are made. DMSO, captan, and thalidomide have been administered to dams, injected between implantation sites in utero, added to preimplantation embryos cultured in vitro, and injected directly into blastocysts. Attempts are also being made to determine the extent of absorption of the test agent by the embryo and its biological half-life.

The potential chemical interactions in our complex environment are multiple and synergistic responses might explain a large part of the still undefined incidence of birth abnormalities. Chemicals which appear innocuous using currently accepted laboratory tests might actually be toxic in combination with other chemical, physical, physiologic, or pathologic factors. Dichlorvos (a widely used insecticide) was studied in three species by several routes alone and in combination with other agents (e.g., alcohol, carbon monoxide, hypothyroidism). This work was conducted jointly through a contract with Dow Chemical Company and intramural efforts. The results are presently being evaluated, but potentiation of toxicity was not obvious. Current investigations focus on a slightly more toxic compound, carbaryl, and additional modifiers (e.g., protein deficiency).

As part of the US-USSR Cooperative Program, Dr. Staples and his coworkers studied the teratogenic potential of two insecticides (Dipterex, Imidan) in the rat. A positive teratogenic response was obtained with Dipterex but not with Imidan. Dipterex is now being studied in additional species.

The world's literature on the teratogenic effect of environmental agents is being organized into a teratology information program which will aid in determining priorities and planning future investigation. Dr. Staples also has organized a group to review environmental chemicals for teratogenic testing and to set priorities for contract screening studies.

Dr. Maurer, in collaboration with scientists in Germany, has also been studying uterine luminal fluid proteins in rabbits to determine their role during early pregnancy and their susceptibility to change in an altered environment. For instance, injection of progesterone before ovulation produces a premature appearance of uteroglobin, a uterine luminal protein, suggesting an asynchronous uterine environment for the developing embryo.

Research efforts of the Developmental Toxicology Section are concerned with selected biochemical and physiological effects of prenatal and/or postnatal exposure to environmental chemicals on fertility, the early conceptus, fetus, and newborn. The reproductive and perinatal parameters studied attempt to elucidate the mechanisms by which environmental agents affect differentiation and development. In vivo and in vitro techniques are utilized. Metabolism and tissue distribution are studied in target and non-target tissues in relation to observed toxic responses. Recent research achievements of sectional work groups can be summarized as follows.

Dr. McLachlan and coworkers have been studying the effects of environmental agents on reproductive tract function using mice. Current studies involve the synthetic estrogen diethylstilbestrol (DES), a common environmental chemical currently used as a livestock food additive and gynecologic medication. Experiments in their laboratory have demonstrated that prenatal exposure to DES adversely affects the reproductive capacity of the female offspring. This effect is dose-related and is due, in part, to the relative inability of the female offspring to ovulate. Likewise, the male offspring are sterile following prenatal exposure to DES. The reproductive tracts in the prenatally-drug exposed offspring have lesions which include metaplastic and/or neoplastic changes. These results may be pertinent to the development of an animal model to study the reproductive tract lesions reported in young women whose mothers had been given DES during pregnancy. Since little is known concerning the physiologic disposition and metabolism of DES during gestation, the fate of ^{14}C -DES in the pregnant mouse was studied. The compound is rapidly cleared from the plasma following intravenous administration. Relative to the plasma, DES accumulates in the maternal liver and, to a lesser extent, in the uterus and fetal liver. The activity of glucuronyltransferase in the maternal and fetal liver helps account for the accumulation of radioactivity identified as DES conjugates. Attempts are being made to utilize a recently developed fetal ovary organ culture system to study direct fetal reproductive tract effects of DES.

Dr. Lee's group has been investigating whether the DNA repair system in mouse germ cells can be utilized as a predictive test system for environmental mutagens. Biochemical and toxic effects of environmental agents on mouse spermatogenesis are also being studied. Enzyme development during spermatogenesis is being determined in an attempt to define "marker" enzymes indicative of normal and abnormal cellular differentiation. The interrelationship between hormone action and ontogeny of testicular enzyme synthesis is also being studied as well as the nature of the blood-testis barrier. Experimental data suggested that monofunctional alkylating agents, administered *in vivo*, cause single strand DNA breaks in spermatogonia. The extent of single DNA breaks is dose dependent and DNA repair time is dependent upon the degree of DNA damage. In contrast, multifunctional alkylating agents do not cause single strand DNA breaks but crosslink between DNA-DNA and/or DNA-proteins. Spermatogenic enzyme studies indicated that hyaluronidase and several glycolytic dehydrogenase enzymes might be useful as "markers" for selective testicular effects of environmental chemicals. Ontogeny of these enzymes was shown to be androgen dependent. The transport of cadmium ions across the blood-testis barrier was found to be an active process which can be inhibited by zinc pretreatment. The permeability characteristics of the blood-testis barrier closely resemble those for the blood-brain barrier with regard to passive diffusion of chemicals.

Dr. Moustafa and coworkers are studying cellular homotypic and heterotypic interactions in chimaeric animals. Results have demonstrated that single blastocyst cells transferred to preimplantation blastocysts (mice and rabbits) resulted in chimaeric animals with additive body weights. Effects on sex chromosomes were evidenced by changes in the sex ratio (favoring males 2:1) and the incidence of sterility (12% and 33% among phenotypic females and males, respectively) among resultant chimeras. The mechanisms of these interactions are being investigated at the cytological, biochemical, and physiological levels. Isolated teratocarcinoma cells, treated or untreated with specific carcinogens and cell-locomotion inhibitors, are also being used in cell transfer studies. This system should provide information regarding mechanisms of defects involving specific cell differentiation. Dr. Moustafa, in collaboration with other NIEHS scientists, is studying chemical and UV radiation induced abnormalities in preimplantation and postimplantation embryos. Morphological damage observed in embryos irradiated during cleavage include fragmentation, incomplete cleavage, pycnosis, and cytoplasmic vacuolization. The effects were dose and time dependent and the number of abnormalities increased with each succeeding cleavage. The percentage of embryos which implanted was greater among surviving embryos from lower dose exposure groups. Current investigations involve the use of preimplantation and postimplantation embryos and teratomas as models for chemically-induced abnormalities in differentiation.

Dr. Lucier and coworkers are concerned with the metabolism and binding of xenobiotics and steroids in relation to reproductive and developmental toxicity. Emphasis is placed on the perinatal development of steroid receptors, glucuronidation pathways, and cadmium binding proteins. Results have shown that guinea pig, rat, and rabbit liver p-nitrophenol glucuronyltransferase (PNP UDPGT) activity about the time of birth, was equivalent to the adult whereas testosterone and β -estradiol glucuronidations were barely detectable at this

time. A single oral dose of 3 $\mu\text{g/kg}$ 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) on the fifth day of gestation had no effect on the hepatic perinatal development of steroid UDPGT, whereas there was a marked and prolonged increase in postnatal PNP UDPGT activities. These data along with kinetic studies suggest that there are at least two microsomal UDP glucuronyltransferases. The postnatal induction of PNP UDPGT, as well as components of the mixed function oxidase system, was shown to be caused both by newborn exposure to TCDD via maternal milk and the activation of an inducing mechanism just prior to birth. Data obtained from solubilized and partially purified enzyme preparations indicated that the induction of PNP UDPGT by TCDD was apparently caused by increased enzyme synthesis. Glucuronidation of DES was shown to be more in the class of steroid glucuronidation than non-steroid glucuronidation supporting the molecular similarity of DES and natural estrogens. Cadmium administered maternally crossed the placenta in relationship to dose and placenta transfer increased with gestational age. The ability of cadmium to induce metallothionein synthesis in maternal liver and/or placenta, and the saturation of this metal binding protein is being further studied.

Scientists in the Biochemical Toxicology Section are engaged in a coordinated effort to determine the basic biological mechanisms through which toxic environmental agents exert their morphologic and biochemical effects in marine and mammalian species and to apply this information to the design of more rapid and effective test procedures to predict toxicity in man. Summaries of the research interests and achievements of Biochemical Toxicology Section scientists follow.

Dr. Woods and coworkers are concerned with factors affecting hepatic heme synthesis during mammalian development. In adults, δ -aminolevulinic acid (ALA) synthetase is the rate-limiting enzyme in the heme biosynthetic pathway. The regulatory properties of this enzyme have been characterized in both adult and fetal liver. Fetal ALA synthetase is not regulated by the end product heme, as is the adult enzyme, but is more highly susceptible than the adult enzyme to inhibition by high cation concentrations and changes in pH and temperature as well as other *in vitro* conditions. These results suggest that ALA synthetase may not be rate-limiting in hepatic heme biosynthesis until it becomes susceptible to regulation by heme, a phenomenon which does not occur until after birth. In addition, these studies indicate that developing liver may be somewhat more susceptible to toxic effects of environmental agents due to immature regulatory mechanisms. Other studies are in progress to consider the biochemical and pharmacologic basis for the laboratory prediction of human responses following exposure to porphyrogenic and/or hematotoxic environmental agents.

Dr. Andrew has determined that the progestational steroid medroxyprogesterone acetate (MPA, Provera ^R), a suspected teratogen in humans, results in offspring with cleft palate when administered to pregnant rabbits. Teratogenicity was not noted in rats or mice even at the maximum tolerated dose. In view of this obvious species difference and the rare occurrence of palatal malformations in the rabbit, the teratogenic mechanisms of MPA are being explored further in rabbits. Dr. Andrew is also developing a program to monitor and assess chemically-induced changes in amniotic and fetal enzymes (and other proteins) for their ability to predict developmental toxicity.

Drs. Woods and Fowler are collaborating in the development of a program to establish biochemical and behavioral toxicity profiles in mammalian and marine species exposed chronically to low levels of various environmental agents, especially heavy metals. Toxicity profiles in lower species will be utilized to derive predictive model test systems for humans. Agents currently under investigation include arsenic and methyl mercury. Plans are being developed to investigate a wide variety of environmentally relevant agents in marine and aquatic as well as mammalian species. Studies of marine organisms allows an estimation of toxicity in the normal setting with natural conditions, a comparison of toxic effects with mammalian species, and a better understanding of the localization and accumulation of these elements by lower animal species.

Dr. Crapo has been studying the relationship between superoxide dismutase (SOD) and pulmonary oxygen toxicity. Rats exposed to 85 percent oxygen for 7 days have been shown to acquire "tolerance" to lethal concentrations of oxygen. This physiological adaptation has been shown to correspond in time with an increase in the SOD in the lungs of these animals. Antibody titrations of SOD have further demonstrated that this increase is in the cytosol form of the enzyme. Investigations are now in progress to further define the biochemical adaptations occurring during exposure to oxygen and to more accurately identify how SOD is changing. Appropriate light and EM histopathology is being used to determine the histological response to oxygen exposure. Alveolar type II cells have been shown to proliferate at the same time that the physiological and biochemical adaptations occur. Because a large number of environmental toxicants may produce their toxic effects by way of free radicals, several of them, including ozone, NO₂, SO₂, and paraquat, are being studied using the same tools developed for the study of oxygen toxicity.

Dr. Fowler is attempting to define the early ultrastructural and biochemical effects of heavy metals and other environmental toxicants on mammalian and marine invertebrate tissues. Subcellular morphologic observations are correlated with tissue concentrations and chemical alterations in blood and viscera. His studies attempt to define the ability of electron microscope techniques to predict early signs of chemical toxicity. Arsenic, cadmium, bismuth, methyl mercury, inorganic mercury, and dioxin are being studied. Combined oxygen electrode and ultrastructural studies were performed on kidneys of rats exposed to arsenate in the drinking water for six weeks. Decreased mitochondrial state 3 respiration was observed in all dose groups, and a dose related increase in swollen mitochondria and dense autophagic lysosomal bodies was observed in the proximal tubules. Ultrastructural studies were conducted on kidneys of rabbits receiving a single injection of cadmium-metallothionein. A dose equivalent to 0.4 mg/kg cadmium produced necrosis of tubule cells; increased numbers of swollen mitochondria and dense granular lysosomal bodies were noted in proximal tubule cells by electron microscopy at lower doses. Females rats were given access to drinking water containing methyl mercury hydroxide for four weeks prior to mating, and fetal and maternal liver mitochondria were studied for changes in ultrastructure, respiratory capacity, and protein synthesis on day 10 of pregnancy. The fetus appeared more sensitive than maternal animals to dose related alterations of mitochondria morphology and function. Aspects of dioxin induced induction of renal microsomal enzyme activities associated with proliferation of the

smooth endoplasmic reticulum was further studied. X-ray microanalysis techniques are being used for localization of heavy metals within cells.

Dr. Kimmel is working on developing a program of behavioral toxicology at the NIEHS. This program will attempt to define methods to determine what environmental stresses produce behavioral alterations in mammals and lower species. Model test systems which simulate and predict behavioral toxicity in humans will be developed and neurochemical mechanisms of behavioral toxicity considered.

Dr. Kimmel is also studying the teratogenic potential and toxic mechanisms of chelating agents, e.g., EDTA, and their interaction with metals, especially with regard to zinc deficiency. EDTA treatment and zinc deficiency produce very similar types of malformations in rats. Gonad dysgeneses was seen in many malformed fetuses. It was impossible to determine the sex of offspring on gross observation of the gonads. Organ size and number of germ cells was greatly reduced in both ovary and testis. EDTA-treated and zinc-deficient fetuses were almost identical in appearance. Postnatal development of offspring of EDTA treated animals was severely affected; survival to weaning and growth rates were reduced.

Dr. Spalding and coworkers are currently working on the development of in vitro mammalian tissue and organ culture test systems which can be utilized for the rapid prediction of potentially toxic environmental agents. Progress has been made regarding the development of a viable tissue or organ culture system of mammalian gonadal tissue. The mechanism by which chemical agents affect oogenesis and the subsequent stages of oocyte maturation and follicular development will be studied using this system. Oocyte maturation in ovary explants taken from 16 day old embryos was followed for 12-14 days in culture. Oocyte maturation in vitro occurred in a manner analogous to maturation in vivo to about 25 days postconception. The effects of temperature and alterations in growth medium on follicular maturation have been established. Attempts to establish consistent and reproducible stages of growth and maturation of fetal and prepubertal ovarian explants will be continued. Another ongoing study seeks to define the interaction of organochlorine compounds with the plasma membrane of mammalian cell grown in vitro, and to determine how this interaction relates to toxicity. Representatives of several different classes of organochlorine compounds, e.g., DDT, chlordane, and 4-chlorobiphenyl, have been studied using L5178Y cells in culture. The effect of these chemicals on transport and phospholipid metabolism among the different membrane-containing organelles of the cell are being studied, and the nature of the interaction with the plasma membrane is being investigated.

Dr. Weissberg has been evaluating in vitro techniques to study toxic hematologic effects of environmental agents. A tissue culture system that allows sustained proliferation and differentiation of bone marrow cells has been developed for the purpose of identifying agents that are toxic to the bone marrow, evaluating the role of metabolism in hematotoxicity, and elucidating biochemical mechanisms of toxicity. Culturing of individual cell types isolated by velocity sedimentation is being evaluated. This technique allows a clear separation of nucleated erythroid cells from cells in the granulocytic series. Bone marrow cells cultured in the liquid system show

sustained cellular proliferation and differentiation. In initial toxicity studies, benzene has been employed as a model agent to apply in vitro techniques to the study of environmental pollutants. A dose related effect of benzene on cellular proliferation of bone marrow culture has been observed.

Dr. Wilson and coworkers have been studying the extent to which modification of plasma membranal structure and function correlate with fetal toxic and/or teratogenic responsiveness. Attempts are being made to characterize the properties and behavior of plasma membranal regulatory enzymes from fetal tissues and compare the behavior of these enzymes with the corresponding adult enzymes. An area of special interest involves the biochemical basis for the occurrence of abnormal ectodermal development about the brain and spinal cord. Aspirin administered to pregnant rats elicits abnormal ectodermal development, e.g., exencephaly, spina bifida, in fetal rats. This response to aspirin is augmented by simultaneous administration of a diet deficient in pantothenic acid and, possibly, essential fatty acids. Grossly observable teratogenic effects are noted when pregnant rats are fed a pantothenic acid deficient diet for several days or a deficient diet with a pantothenic acid antimetabolite for a shorter period. Biochemical studies involve efforts to determine the roles of prostaglandins and/or cyclic nucleotides in regulating behavior and/or development of embryonic cells. Initial studies will examine prostaglandin synthetases and/or nucleotide cyclases of uterine, placental, and several fetal tissues.

Dr. Jurgelsky has continued his studies to evaluate the marsupial as a model for carcinogenesis. The autopsy phase of this project has been completed. Ethyl nitrosourea, a known potent carcinogen, was administered orally as a single dose (100 mg/kg) to opossums at ten different stages of development. Eye tumors and several other unusual tumors have been found in ENU-treated animals which might be of interest to the National Cancer Institute.

In the coming year, the Environmental Toxicology Branch will attempt to further narrow the range of branch research efforts and concentrate resources more effectively. Major areas of interest include environmental factors affecting germ cell development, teratology (early effects of gestation chemical exposure), transplacental toxicology (functional effects of gestational chemical exposure), environmental factors involved in prematurity, rapid toxicity test systems, mechanisms of target organ toxicity, and effects of environmental agents on learning and behavior. The techniques and scientific skills represented by these areas will also be available to support the NIEHS's energy related health effects research efforts.

1. Environmental Toxicology Branch
- 2.
3. Research Triangle Park, N.C.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

PROJECT TITLE: "Effect of Environmental Components on Mammalian Ontogenesis After Parental Exposure"

PREVIOUS SERIAL NUMBER: NIEHS-ET-003

PRINCIPAL INVESTIGATORS: R. E. Staples, Ph.D., F. D. Andrew, Ph.D., C. A. Kimmel, Ph.D., M. H. Lu, Ph.D., R. R. Maurer, Ph.D., and U. Schneider, D.V.M.

OTHER INVESTIGATORS: E. H. Floyd, R.T., R. G. Kellam, B.S., C. D. Sloan, M.S., and P. E. Watkins, B.A., M.T.

COOPERATING UNITS: Research Triangle Institute (Procurement Contract: NIH-74-C-390). Environmental Biophysics Branch (Noise).
Environmental Biometry Branch

MAN YEARS

Total:	6.2
Professional:	3.6
Other:	2.6

PROJECT DESCRIPTION

OBJECTIVE: To determine the developmental toxicity and teratogenic potential of single or combined environmental components in mammals by application of the agents to the dam.

METHODS EMPLOYED: Test agents were given to pregnant females of three species, mouse, rat, and rabbit by two or more routes, e.g., per os, subcutaneous, intrauterine, for various periods of ontogenesis. Doses were selected on the basis of preliminary study. Those which produced a given pharmacological response, (maximum tolerated dose, comparable blood levels, 50% fetal mortality, etc.) were selected. Stresses or disease states that may be elicited before mating were also studied for teratogenic potential either alone or in combination with other environmental components. An attempt was made to compare the sensitivity of various approaches. Qualitative or quantitative differences or similarities in teratogenic response were noted. Resulting offspring were examined for alterations in the incidence of external, visceral, and skeletal changes shortly before term.

MAJOR FINDINGS: Analogs of Provera (medroxyprogesterone acetate) and related steroids were tested in pregnant rabbits to identify structure-activity relationships. Of those steroids tested, only triamcinolone acetonide (0.3 mg/kg/day for 3 days, s.c.) produced a spectrum of embryotoxicity (including teratogenicity) analogous to that seen after Provera administration (3 mg/kg/day for 3 days, s.c.). In contrast, Provera-related embryotoxicity was not seen in mice or rats after the highest dose and longest exposure tested (3000 mg/kg/day for 9 days, s.c.).

Pregnant mice (CF₁S strain) exposed to 100 decibels of white noise for 6 min of every half hour on Days 3 through 6, 7 through 10, or 11 through 14 of gestation had more resorption of their young but no indication of teratogenicity. In rats exposed to noise on Days 6 through 15 (same regimen as above) no teratogenic or embryolethal effects were observed.

Ethylene thiourea (ETU), a fungicide, was administered to intact and thyroidectomized female rats to determine whether the previously reported teratogenicity of the chemical would be inhibited in the absence of the thyroid gland. Malformed fetuses were present in the thyroidectomized and intact groups which indicated that the teratogenic effect of ETU is not mediated by the thyroid; a possibility advanced in the literature.

Dipterex and Imidan, two organophosphate insecticides that are used in the United States, were studied for teratogenic potential in the rat as a part of the USA-USSR Scientific Cooperation Program. Imidan was not found to be teratogenic after administration by diet or gavage from Days 6 through 15 of gestation at dosages up to the maximum tolerated, but under the same regimen. Dipterex was found to be teratogenic when administered by diet at daily dose levels of 432 mg/kg or more; a high dose relative to human exposure. Similar teratogenicity was achieved by administration by gavage if the daily dosage was divided to prevent toxicity to the dam. Dipterex was not known to have teratogenic potential previously.

³⁵S-Captan was administered to the uterine horns of mice on Day 12 of pregnancy to determine the distribution and persistence of radioactive label as a continuation of studies undertaken last year with Captan. Data collected to date indicate that labelling of the fetuses persisted for at least 24 hours after administration, and traversed anteriorly as well as posteriorly from the injection site. Unlabelled Captan was administered to the uterine horns of rabbits on Day 8 of gestation at 0, 300, and 3000 µg/horn. Embryonic mortality was increased in a dose-related manner but teratogenicity was not noted.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Through the efforts described, the teratogenic potential of environmental components are evaluated. The teratogenic response of various species to some of the agents being tested in vivo is compared to the response after exposure of pre-implantation embryos during culture. Our hope is that a means of testing can be derived that will improve extrapolation of experimental results to man.

Modern man is exposed continuously to a multitude of physical stresses and chemical insults, yet most agents tested for teratogenic potential are still being studied as single entities even though synergistic effects in animals have been documented. One of the goals of this study is to determine the likelihood of synergism of teratogenic potential with combinations of environmental components to which the housewife might reasonably be exposed. Environmental components to be tested are first studied individually and then, depending upon the information gained, combinations of components will be tested.

PROPOSED COURSE: Dipterex will be administered to hamsters and mice to determine if the positive teratogenicity seen in the rat is unique or if it extends to other species.

To determine the role of the thyroid gland in postnatal maturation and subsequent reproductive capability, rat pups are to be thyroparathyroidectomized surgically on Days 1, 7, 14, 21, and 28 after birth. Preliminary data indicate that maturation and fertility is severely affected if the thyroid is removed before Day 15 postpartum.

Other agents, e.g., epoxide, asbestos, will be administered to pregnant females orally or by intrauterine injection to determine developmental toxicity.

KEYWORD DESCRIPTORS: teratology; developmental toxicity; ethylenethiourea; Dipterex; Imidan; Captan; intrauterine application; rabbit; mouse; rat; noise; Provera; medroxyprogesterone acetate

PUBLICATION

Staples, R.E., Kellam, R.G., and Haseman, J.K.: Developmental toxicity in the rat after ingestion or gavage of organophosphate pesticides (Dipterex, Imidan) during pregnancy. Environ. Health Persp. (in press).

Project No.: Z01 ES 70021-03 ETB
1. Environmental Toxicology Branch
2.
3. Research Triangle Park, N.C.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

PROJECT TITLE: "Survival and Development of Mammalian Gametes and Embryos
After In Vitro Exposure to Selected Chemicals and Conditions"

PREVIOUS SERIAL NUMBER: NIEHS-ET-004

PRINCIPAL INVESTIGATORS: R. E. Staples, Ph.D., R. R. Maurer, Ph.D., and
L. A. Moustafa, Ph.D.

OTHER INVESTIGATORS: U. Schneider, D.V.M., P. E. Watkins, B.A., M.T.,
E. H. Floyd, R.T., and W. D. Willis

COOPERATING UNIT: Research Triangle Institute (Procurement Contract:
NIH-74-C-390)

MAN YEARS

Total:	2.9
Professional:	1.7
Other:	1.2

PROJECT DESCRIPTION

OBJECTIVE: To determine the effects of various chemical and physical entities, acting alone and in combination, on subsequent development of mammalian gametes and embryos after exposure in vitro or intrauterine.

METHODS EMPLOYED: Embryos are recovered from donor animals and cultured in vitro for varying periods. Chemicals or conditions being tested are generally applied during the culture period. Some preimplantation embryos are transferred to recipient females while others are examined for morphologic or chemical changes. Transferred embryos are usually examined near term for external, visceral, and skeletal malformations, but when indicated, they may be studied at earlier stages of gestation or postnatally.

Cultured preimplantation embryos were also injected with or exposed to test agent(s) (e.g., thalidomide), embryos transferred into recipient females which were sacrificed for teratological evaluations. Radiolabeled thalidomide was used to trace its uptake and distribution in embryonic tissues after in utero administration.

MAJOR FINDINGS: Asbestos was administered to mouse blastocysts for 4 hours during culture on the fourth day of development at dose levels of 0, 1, 10, and 100 $\mu\text{g/ml}$ of medium. Structural malformations associated with asbestos administration were not detected among the offspring that resulted after transfer of the blastocysts to recipient females.

Captan was administered to mouse blastocysts for 4 hours during culture on the fourth day of development at dose levels of 0, 25, 50, 67, 85, and 100 ng/ml of medium. Subsequent development of the blastocysts after transfer to recipient females revealed dose-related fetal toxicity. Malformations were found among the fetuses that were exposed to the 25 and 50 ng doses of Captan as blastocysts. In contrast, no malformations were observed among those blastocysts exposed to higher dose levels. Rabbit blastocysts similarly exposed to Captan for 4 hours on Day 6 of gestation demonstrated embryo toxicity but not teratogenicity after transfer to pseudo pregnant females.

Rabbit and mice blastocysts were micromanipulated or cultured with thalidomide, and transferred into untreated foster mothers with no apparent adverse effects on development. Accumulated data indicated that the higher thalidomide doses used were embryolethal only; lower doses did not induce any significant morphological malformations. Thalidomide was detected in embryonic tissues as well as blastocoelic fluids.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Techniques permitting development of preimplantation embryos *in vitro* with retention of viability to term after transfer to the uteri of recipient females makes possible direct exposure of mammalian embryos to test agents. This permits investigators to distinguish between direct and indirect effects of environmental insults on embryo development. Information will also be gained that will increase our understanding of embryonic requirements for development. Results of local exposure are compared to those seen following administration to the dam or to the uterus. Perhaps variability of response to teratogens can be reduced among species if maternal pharmacokinetics are circumvented. Teratogenic agents might also be identified after local application of only a small fraction of the dose currently required by in vivo tests.

PROPOSED COURSE: Asbestos fiber penetration into mouse blastocysts during culture will be determined.

Additional mouse blastocysts are to be exposed to Captan at 0, 10, 20, and 25 $\mu\text{g/ml}$ to determine whether less toxic doses will enhance the teratogenic response. Rabbit blastocysts will be cultured in ^{35}S -Captan to determine degree of penetration of the radioactive label.

It is also proposed to expose rat blastocysts during culture to the organophosphate insecticide, Diptorex, to determine whether the teratogenic response seen after oral administration to the dam can also be elicited by direct exposure of the blastocysts earlier in gestation.

KEYWORD DESCRIPTORS: mammalian embryo; rat; mouse; rabbit; asbestos; Captan; thalidomide; blastocyst; morula; embryo transfer; embryo culture; malformations; teratology

Project No.: Z01 ES 70040-04 ETB
1. Environmental Toxicology Branch
2.
3. Research Triangle Park, N.C.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

PROJECT TITLE: "Low Temperature Storage of Mammalian Embryos"

PREVIOUS SERIAL NUMBER: NIEHS-ET-006

PRINCIPAL INVESTIGATOR: R. R. Maurer, Ph.D.

OTHER INVESTIGATORS: U. Schneider, D.V.M., J. K. Haseman, Ph.D. and
L. C. Ulberg, Ph.D., Department Animal Science,
North Carolina State University

COOPERATING UNIT: Environmental Biometry Branch

MAN YEARS

Total:	0.6
Professional:	0.5
Other:	0.1

PROJECT DESCRIPTION

OBJECTIVES: (1) To establish methodology for the storage of viable mammalian embryos, (2) to assess the effects of freezing and low temperature storage on subsequent in vitro and in vivo development, (3) to determine if mammalian embryos of various species respond similarly to freezing and low temperature storage, and (4) to provide a bank of stored embryos.

METHODS EMPLOYED: Embryos are recovered by conventional methods from mice on Day 3 or 4 of gestation and from rabbits on Day 2 or 3 of gestation. The embryos are washed with and placed in a phosphate buffered saline solution fortified with pyruvate, bovine serum albumin and DMSO. Embryos are frozen slowly (0.5 to 1.5°C/min), and the temperature gradually reduced to -100°C before the embryos are placed in liquid nitrogen (-196°C). After various periods of storage, the embryos are thawed slowly (4 to 50°C/min), washed and placed in culture for 48 hours. After culturing, the resulting mouse blastocysts are transferred to the uterus of Day 3 recipient mice. Some of the recipients are allowed to parturate. The fetuses from the remaining recipients are removed by Cesarean section one or two days before normal deliver and examined for teratological changes. Rabbit morula are transferred immediately after thawing to Day 2 recipients for subsequent evaluation of in vivo development.

MAJOR FINDINGS: Rabbit: An optimization procedure was used to determine conditions for freezing morula stage embryos from Dutch-Belted rabbits. The optimal conditions found were: 2.0 M DMSO; 10.7 minutes needed to equilibrate with cryoprotective agent; 1.0°C/minute cooling rate; -100°C temperature at which to stop slow cooling and 17.0°C/minute warming rate. Of embryos frozen under these conditions, 83% developed into blastocysts during subsequent culture. In contrast to the 8-celled embryo, cooling rate was not as important for the morula stage embryos. DMSO concentration and equilibration time were the most important factors for the morula stage. Viable fetuses developed from 26% of all frozen and thawed morula transferred to surrogate females. Fewer morula from New Zealand white rabbits developed in vivo than for morula from Dutch-Belted rabbits; hence each strain may require a different set of conditions to obtain optimal postthaw development.

Mouse: Blastocysts readily freeze with 80 to 90% postthaw survival in culture. Warming the embryos between -10 and 0°C appears to be very important in obtaining high postthaw development in vitro.

Swine: Eight-celled and morula staged embryos did not survive freezing using conditions which allowed mouse and rabbit embryos to continue to develop after freezing and thawing.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Human spermatozoa are frozen and banked for routine clinical inseminations as well as for vasectomized males who may at a later time desire children. The eventual storage of human embryos must also be considered. The main cryoprotective agent is DMSO, which is known to be teratogenic at high levels. Therefore, the potential hazard should be assessed through use of animal models.

The freezing and storage of embryos can aid experimentation by providing a readily available source of material, preserving mutant strains of species, and providing a more precise measurement of genetic drift as well as changes in susceptibility to various environmental insults in mammalian species and strains. Proper preservation of embryos will reduce preparation artifacts, thereby aiding subcellular studies. The general technique being developed can also provide further information regarding the freezing and banking of human tissues and organs.

PROPOSED COURSE: Species and strain differences and freezing at other stages of development will be investigated.

KEYWORD DESCRIPTORS: low temperature storage; mammalian embryos; freezing embryos; in vitro development, in vivo development; mouse embryos; rabbit embryos; methodology

PUBLICATIONS

Bank, H. and Maurer, R.R.: Survival of frozen rabbit embryos. Exp. Cell Res. 89: 188-196, 1974.

Bank, H. and Maurer, R.R.: Cryogenic preservation of eight-celled and morula staged rabbit embryos. ICLA Proceedings. Biological Aspects on Freeze Preservation of Mouse Strains (in press).

Project No.: Z01 ES 70060-02 ETB

1. Environmental Toxicology Branch
- 2.
3. Research Triangle Park, N.C.

PHS-NIH

Individual Project Report

July 1, 1974 through June 30, 1975

PROJECT TITLE: "Effect of Prenatal Exposure to Foreign Chemicals on the Development of Reproductive Tract Function in Mammals"

PREVIOUS SERIAL NUMBER: NIEHS-ET-002

PRINCIPAL INVESTIGATOR: J. A. McLachlan, Ph.D.

OTHER INVESTIGATORS: H. C. Shah, Ph.D. and J. W. Spalding, Ph.D.

COOPERATING UNITS: B. C. Bullock, Ph.D., Bowman-Gray School of Medicine

MAN YEARS

Total:	3.4
Professional:	2.0
Other:	1.4

PROJECT DESCRIPTION

OBJECTIVES: (1) To evaluate the effects of prenatal exposure to environmental chemicals on the subsequent reproductive capacity of the offspring, (2) to investigate the mechanisms involved in the production of subfertility in females as a result of their in utero exposure to foreign chemicals, (3) to assess the transplacental carcinogenic potential of these compounds, (4) to study the physiologic disposition and metabolism of these compounds in the pregnant animal and fetus, (5) to determine if prenatal exposure to environmental agents can alter the cytoplasmic receptors for steroid hormones in reproductive tract tissue, and (6) to develop and utilize the effects of environmental chemicals on the fetal ovary and reproductive tract in vitro.

METHODS EMPLOYED: The most sensitive measurement of female gonadal function is her total reproductive capacity as determined by repetitive forced breeding techniques. In order to assess the functional status of the female gonad, these techniques were coupled with the determination of ovarian periodicity, quantity and morphology of ova obtained from the female reproductive tract, and histopathologic evaluations of the ovary including quantitative determinations of the various follicular classes. Biochemical, physiologic, and morphologic procedures were used which include polyacrylamide gel electrophoresis, radioimmunoassay, histochemistry, autoradiography, and microdissection. Organ culture techniques were developed to maintain explants of fetal ovaries and reproductive tracts.

MAJOR FINDINGS: The synthetic estrogen, diethylstilbestrol (DES), is a common environmental chemical currently used as a livestock food additive and gynecologic medication. Experiments in our laboratory have demonstrated that prenatal exposure to DES adversely affects the reproductive capacity of the female offspring. This effect is dose-related and is due, in part, to the relative inability of the female offspring to ovulate. Likewise, the male offspring are sterile following prenatal exposure to DES. The reproductive tracts in the prenatally-drug exposed offspring have lesions which include metaplastic and/or neoplastic changes. These results may be pertinent to the development of an animal model to study the reproductive tract lesions reported in young women whose mothers had been given DES during pregnancy.

Since little is known concerning the physiologic disposition and metabolism of DES during gestation, the fate of ^{14}C -DES in the pregnant mouse was studied. The compound is rapidly cleared from the plasma following intravenous administration. Relative to the plasma, DES accumulates in the maternal liver and, to a lesser extent, in the uterus and fetus. The activity of glucuronyl-transferase in the maternal and fetal liver helps account for the accumulation of radioactivity identified as DES conjugates in these two organs.

Attempts are being made to utilize the fetal ovary organ culture system which has been developed to study direct fetal reproductive tract effects of DES.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Although many compounds are continuously introduced into our environment, few of them have been examined for their potentially toxic effect on reproduction and development. Virtually nothing is known about the effect of prenatal exposure to common drugs and chemicals on the postnatal development of the offspring. The fact that no division of oocytes occurs postnatally in man or laboratory rodents makes the process of oogenesis especially susceptible to chemical intervention during the prenatal period. However, the effects of such in utero drug exposure may not become evident until much later in the animal's life when sexual maturity is reached. Given the possibility of long-term genetic damage to the developing oocyte or transplacental carcinogenetic changes in the reproductive tract, reduced fertility in the offspring may be only the most obvious consequence of prenatal exposure to environmental chemicals.

PROPOSED COURSE: The studies dealing with the total reproductive capacity of mice exposed to diethylstilbestrol in utero will be continued with emphasis placed on investigating the mechanisms underlying these observations. Experiments to determine sensitive periods and structure-activity relationships involved in this effect will be continued. Moreover, studies of steroid receptor proteins in prenatally DES-exposed females are being attempted. The mouse fetal gonad organ culture system will be refined and expanded to include co-culturing of fetal gonads and genital tract tissue.

KEYWORD DESCRIPTORS: prenatal exposure to foreign chemicals; reproductive tract function; reproductive capacity of the offspring; transplacental carcinogenic; physiologic disposition and metabolism; cytoplasmic receptors for steroid hormones; fetal ovary; in vitro; diethylstilbestrol; animal model

Project No.: Z01 ES 70080-02 ETB

1. Environmental Toxicology Branch
- 2.
3. Research Triangle Park, N.C.

PHS-NIH

Individual Project Report

July 1, 1974 through June 30, 1975

PROJECT TITLE: "The Study of Toxic Effects of Environmental Chemicals on Spermatogenesis"

PREVIOUS SERIAL NUMBER: NIEHS-ET-001

PRINCIPAL INVESTIGATOR: I. P. Lee, Ph.D.

OTHER INVESTIGATOR: R. S. Shen, Ph.D.

COOPERATING UNIT: Hazleton Laboratories, Inc.

MAN YEARS

Total:	3.6
Professional:	2.0
Other:	1.6

PROJECT DESCRIPTION

OBJECTIVES: (1) To investigate whether the DNA repair system in mouse germ cells can be utilized as a predictive test system for detection of environmental mutagens; (2) to study biochemical and toxic effects of environmental agents on mouse spermatogenesis using both velocity sedimentation cell separation and serial mating techniques; (3) to define enzyme development during spermatogenesis and attempt to develop useful "marker" enzymes to indicate normal cellular differentiation and toxic effects of environmental agents on mouse spermatogenesis (4) to characterize the interrelationship between hormone action and repression of testicular enzyme synthesis; and (5) to investigate the nature of the blood-testis barrier and the penetration of environmental chemicals to rat spermatogenic cells.

METHODS EMPLOYED: In vivo DNA repair was studied by labeling spermatogonial DNA with repeated i.p. administration of tritiated thymidine to 7 to 10 day old rats. Test mutagens were administered and seminiferous tubules were isolated. Spermatogenic cells were separated from tubules by tryptic digestion. Elution profiles defining DNA molecular size were obtained by alkaline sucrose elution.

Dominant Lethality. Studies using male mice and rats were conducted using the following compounds: sodium tetraborate (Borax), AF₂ (food additive) and nitrous oxide (anaesthetic agent).

Isolation of Spermatogenic Cells by Elutriation Centrifugation. Seminiferous tubules from adult mice were isolated and minced. Minced tubules were incubated with trypsin and tryptic action was stopped by addition of a trypsin inhibitor. Spermatogenic cells were washed and centrifuged three times after filtering through lens paper. One hundred to five hundred million cells were loaded in an elutriative rotor and 0.5% albumin containing Dulbecco-phosphate buffer was allowed to flow through the elutriative chamber at varying speeds to achieve counter current cell separation. Six spermatogenic cell types were separated in sufficient number to determine their "marker" enzymes.

Determination of Spermatogenic Cell Enzymes. Hyaluronidase (H), sorbitol dehydrogenase (SDH), lactic dehydrogenase-X (LDH-X), alpha glycerophosphate dehydrogenase (GPDH), malate dehydrogenase (MDH) and glyceraldehyde-3-phosphate dehydrogenase (G-3-PDH) were assayed in both seminiferous tubules and isolated spermatogenic cells using standard methods. In order to study hormonal regulation of enzymes, specific activities were studied in relation to chronological age and the morphological development of the germ cells. Results were compared with spermatogenic enzyme development in hypophysectomized mice and hypophysectomized mice supplemented with androgens.

Preparation of Rat Testis for Blood-Testis Barrier. Efferent ducts of rat testes were ligated 18 hours before cannulating the rete-testis using micro-capillary tubes. Rete-testis fluid was collected and cadmium was administered to study its permeability. Constant infusion of cadmium was used to establish blood levels and to characterize the permeability of cadmium across the blood-testis barrier. Effects of zinc on cadmium transport were also studied.

MAJOR FINDINGS:

(1) DNA Repair in Spermatogenic Cells.

a. Chemical Mutagens (Monofunctional Alkylating Agents). In vivo exposure of spermatogenic cells to varying doses of methylmethane sulfonate, cyclophosphamide and procarbazine caused DNA breaks in spermatogonial cells but not in spermatozoa. The extent of single strand breaks was dose dependent and DNA repair time depended upon the degree of DNA damage. The rate of single strand DNA breaks was greatest the first hour after MMS administration. DNA repair times for 22.5, 45 and 90 mg MMS/kg were 45, 72 and 96 hours, respectively. Unlike MMS, cytoxan and procarbazine DNA breaks occurred much slower but the extent of DNA damage was far greater. During the first few hours after administration of these compounds, no DNA breaks occurred and maximal single strand DNA breaks were observed after 16 hours. Spermatogonial DNA repair times for cytoxan and procarbazine treated mice were 96 hours and 168 hours, respectively.

b. Other Chemical Mutagens (Multifunctional Alkylating Agents). Busulfan, triethylene melamine (TEM), and mitomycin C showed completely different effects on DNA in spermatogonial cells. Unlike monofunctional alkylating agents, these compounds did not cause single strand DNA breaks but they did produce either cross-linking between DNA strands and/or between DNA and nuclear proteins (e.g. histone and nonhistone proteins). Alkaline sucrose elution analysis indicated that intercalated DNA was retained in the filter better than normal DNA. The

extent of DNA retention might be a useful tool for identifying classes of mutagens.

c. Dominant Lethality Studies. Boron, AF_2 and nitrous oxide had no mutagenic effect on either rats or mice. However, high levels of borax (500, 1000 and 2000 ppm per day as boron equivalents for 30 and 60 days) resulted in antispermatogenic effects in rats. The duration of infertility was dose dependent and correlated positively with increased plasma FSH levels. Both "marker" enzymes and morphological changes induced by boron indicated an initial effect on spermatids similar to hypophysectomy.

(2) Spermatogenic Cell Enzymes. H, SDH, LDH-X, GPDH, MDH and G-3-PDH were assayed in relation to chronological age and to morphological stages of spermatogenesis. Prior to puberty, only MDH and G-3-PDH were detectable. However the other enzymes rose very rapidly coincident with appearance of early spermatids and increases in testosterone concentrations. On the other hand, MDH and G-3-PDH activities were markedly reduced. Specific activities of H, LDH-X, SDH, and GPDH were maximal in 40 day old mice. Hypophysectomy at 50 days caused decreases of H, SDH, LDH-X, and GPDH activities with coincident disappearance of spermiogenic cells, whereas MDH and G-3-PDH increased to prepubertal levels. Enzyme activity changes can be prevented by administering testosterone to hypophysectomized mice. These findings suggest that some enzymes are closely regulated by androgens and might be useful as markers for different stages of spermatogenesis.

(3) Blood-Testis Barrier. The passage of cadmium across the blood-testis barrier was found to be an active process which can be inhibited by zinc pretreatment. Cadmium concentrations in rete-testis fluid were 20 to 30 times higher than that in unbound plasma during constant cadmium infusion.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Studies of toxic effects of environmental agents on reproduction in the past centered largely on teratogenicity and dominant lethal studies. Few investigators have been concerned with the effects of chemicals on fertility and mechanisms of reproductive toxicity. Characterization of the DNA repair system in spermatogenic cells will facilitate the evaluation of dominant lethality. Studies of spermatogenic enzymes associated with spermatogenic cell differentiation and aging will not only help to explain the mechanism of toxic effects, but will also be useful as biochemical "markers" to predict the type of spermatogenic cells that are damaged by toxic agents. Studies of the blood-testis barrier are important to the understanding of how various environmental agents enter the testis and exert toxic actions on germ cells. These types of biochemical and physiological studies are essential in assessing the potential hazards of environmental chemicals to male reproductive function as well as their mutagenic potential at the germ cell level.

PROPOSED COURSE: Studies will continue to determine the extent of DNA germ cell damage caused by various environmental chemicals and to assess potential hazards to man. Further development of biochemical tools will be attempted to study not only the mechanism of action of toxic agents but also the potential use of these techniques for screening environmental chemicals.

KEYWORD DESCRIPTORS: DNA repair; germ cells; environmental agents; spermatogenesis; "marker" enzymes; blood-testis barrier

PUBLICATIONS

Lee, I.P. and Dixon, R.L.: Effects on mercury on spermatogenesis studied by velocity sedimentation cell separation and serial mating. J. Pharmacol. Exp. Ther. (in press).

Okumura, K., Lee, I.P., and Dixon, R.L.: Permeability of selected drugs and chemicals across the blood testis barrier of the rat. J. Pharmacol. Exp. Ther. (in press).

Dixon, R.L., Okumura, K., and Lee, I.P.: Studies of the blood testis barrier in rats. Excerpta Medica (in press).

Dixon, R.L. and Lee, I.P.: Methods of assess reproductive effects of environmental chemicals--studies of cadmium-boron administered orally. Environ. Health Persp. (in press).

Project No.: Z01 ES 70100-03 ETB

1. Environmental Toxicology Branch
- 2.
3. Research Triangle Park, N.C.

PHS-NIH

Individual Project Report

July 1, 1974 through June 30, 1975

PROJECT TITLE: "Studies of Homotypic and Heterotypic Interactions in Chimaeric Mammals"

PREVIOUS SERIAL NUMBER: NIEHS-ET-009

PRINCIPAL INVESTIGATOR: L. A. Moustafa, Ph.D.

OTHER INVESTIGATOR: W. D. Willis, B.S.

COOPERATING UNIT: None

MAN YEARS

Total:	1.6
Professional:	0.6
Other:	1.0

PROJECT DESCRIPTION

OBJECTIVES: (1) To develop a system for determining the role and fate of known transplanted single cell(s) (at a definite stage of differentiation and/or treatment) in the development of host blastocysts (rabbits and mice); (2) to determine the type and degree of cellular interactions in the resultant chimaeras; and (3) to investigate the effects of selected treatment of pre-implantation embryos and/or selected cells, which are to be transplanted, upon subsequent embryonic development.

METHODS EMPLOYED: Experiments were performed in three specific areas: (1) chimaerism was induced by a micrurgical technique (Moustafa, L.A. and Brinster, R. L., J. Exptl. Zool., 181: 181, 1972) in which specific types and numbers of cells were isolated and transplanted into preimplanted mammalian embryos; (2) the complex homotypic and heterotypic (cells of like and unlike developmental history and properties, respectively) interactions in the resultant chimaeric embryos were investigated by observing the cell itself (e.g., pigmentation layer of the retina) and/or the cell product (such as hair pigment or electrophoretic enzyme variants); and (3) determination of the concentration and period of exposure of selected agent(s) (e.g., Cytochalasin B) which inhibit active embryonic cell locomotion and/or control of cleavage. The compound was added to the culture system or injected micrurgically into the mammalian embryos. In all procedures, micromanipulated embryos were cultured until transferred into recipient females or for developmental studies

in vitro. Recipients were scheduled for Cesarean section on the last day of gestation. Fetal viability was recorded, gross congenital malformations noted, and live fetuses were placed with lactating foster mothers which had given birth within the previous 24 hours. Genetic-phenotypic determination of the animals were investigated at maturity.

MAJOR FINDINGS: Embryonic single cells were successfully transplanted into preimplantation intact rabbit blastocysts. Phenotypic differences in the eye, skin, and coat color evidenced successful participation of transplanted cells in host organogenesis. Additional indicators of successful transplantation were: (1) disproportionate sex ratio favoring the male among the experimental young 66.6:33.5 ♂:♀ ($P < 0.05$); (2) incidence of sterility among resultant chimaeras was 12% and 33% among phenotypic females and males, respectively. Among chimaeras which produced young, overt signs of germ cell chimaerism were found in 29% and 30% of the litters of chimaeric females and males, respectively. These results (effects) seem to occur even in animals that are phenotypically indistinguishable from the recipient strain. The possibility of "artificial hybridization" was indicated by the changes in body weight of resultant chimaeras after changing the origin of the transplanted cells.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The analysis of genetic chimaeras has allowed inferences to be made about several otherwise elusive aspects of development including cell determination, division rates, lineage and deployment, and the sizes of pools of precursor cells destined to form particular organs and tissues. Of particular concern is whether a transplanted cell from an embryo with high resistance to an environmental agent would somehow transmit this desideratum to a susceptible recipient embryo; also, whether an auto-immune disease of an embryo can be inhibited by transplanting specific types of cells.

PROPOSED COURSE: After obtaining a suitable number of chimaeric mammals, complete studies of host-transplanted cell interactions and animal phenotypes will be conducted. In addition, we will investigate the possibility of producing an interspecific chimaera, where antigenic differences between the two cell populations may be detected during all developmental stages. Isolated teratocarcinoma cells (treated or untreated with specific carcinogens) will be utilized to produce chimaeras. Resultant chimaeras will be investigated cytologically, histochemically and biochemically, to study the chemical effects on teratocarcinoma cell differentiation as well as to investigate the biochemical and genetic mechanisms that control teratogenesis.

KEYWORD DESCRIPTORS: homotypic and heterotypic interactions; chimaeric mammals; differentiation; microsurgical technique; cell transfer; preimplanted mammalian embryos; culture system; congenital malformations; blastocysts culture; sex ratio; sterility; teratocarcinoma; carcinogenesis

Project No.: Z01 ES 70120-04 ETB
1. Environmental Toxicology Branch
2.
3. Research Triangle Park, N. C.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

PROJECT TITLE: "Metabolism and Binding of Steroids and Xenobiotics in Developing Systems"

PREVIOUS SERIAL NUMBER: NIEHS-ET-010

PRINCIPAL INVESTIGATOR: G. W. Lucier, Ph.D.

OTHER INVESTIGATORS: B. R. Sonawane, Ph.D. and O. S. McDaniel

COOPERATING UNITS: G. Nordberg, M.D. and M. Nordberg, Karolinska Institute, Stockholm Sweden; G. E. R. Hook, Pharmacology Branch, NIEHS

MAN YEARS

Total:	3.6
Professional:	3.0
Other:	0.6

PROJECT DESCRIPTION

OBJECTIVES: (1) To investigate possible enzyme multiplicity and enzyme specificity for glucuronyltransferase during perinatal development in different tissues, for example, determine whether there are different glucuronyltransferases responsible for the glucuronidation of steroids and exogenous chemicals; (2) to characterize conjugation pathways for hormonally-active xenobiotics; (3) to determine if and how environmental chemicals alter the normal developmental pattern of glucuronyltransferase; (4) to characterize the biochemical indicators that are associated with the development of conjugative enzyme activity; (5) to study the role of steroid receptors in developmental and reproductive toxicity; (6) to study the role of steroid metabolism in developmental and reproductive toxicity; and (7) to study the role of placental transfer and placental and fetal binding of cadmium in relation to fetotoxicity and teratogenicity.

METHODS EMPLOYED: Microsomes were prepared using standard procedures. Rapid radiometric techniques were developed in this laboratory to measure conjugative enzyme activity in vitro, based on the partition of non-polar compounds into non-aqueous scintillation fluid. Substrates used for glucuronyltransferase assays were 1-naphthol, p-nitrophenol, testosterone, estrone, estriol, estradiol, diethylstilbestrol, methylumbelliferone, and phenolphthalein. Validities of

colorimetric methods for assaying enzyme activity were determined by using radiolabeled substrates and resolving product from substrate on DEAE-cellulose columns, Sephadex A-25 columns, florisil columns, and thin layer plates. In the glucuronyltransferase developmental studies, at least 15 different ages were used so that sudden fluctuations in enzyme activity could be detected and placental activities measured at many gestational periods. Tissues studied were liver, lung, kidney, small intestine, placenta, uterus, and vagina. Species used were the rat, rabbit, guinea pig, and mouse.

Cytoplasmic and nuclear receptors were measured in target tissues using sensitive microassays. Binding of steroids and xenobiotics to chromatin and DNA were investigated using standard procedures.

¹⁰⁹ Cadmium was injected i.v. to pregnant rats at the 12th, 16th, and 20th day of gestation and maternal liver, maternal blood, placental, and fetal cadmium levels measured 24 hours after treatment. The properties of binding proteins were studied according to M.W., amino acid content, and isoelectric focusing properties.

MAJOR FINDINGS: The perinatal development of hepatic glucuronyltransferase (GT) in rats was studied using testosterone (T), estradiol (E₂), diethylstilbestrol (DES), p-nitrophenol (PNP), 4-methylumbelliferone (MUB), phenolphthalein (P), and 1-naphthol (N) as substrates. Glucuronidation of all substrates was first evident at the 18th day of gestation with the exception of T-GT which was first evident on the day prior to birth. PNP-, N-, and MUB-glucuronidation rates exhibited a marked developmental peak about the time of birth so that enzyme activity at birth exceeded adult levels. Glucuronidation rates of these substrates decreased to adult levels by three weeks after birth. In contrast, E₂-, T-, DES-, and P- glucuronidation rates did not exhibit a developmental peak about the time of birth and glucuronidation rates of these substrates reached adult levels by one week after birth. 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) is an extremely toxic contaminant of some organochlorine compounds and is also teratogenic. We have shown it to be a potent inducer of glucuronyltransferase and other microsomal enzymes in adult rats. For these reasons we were interested in studying effects on fetal microsomal enzymes following maternal exposures. After pregnant rats were administered 3 µg TCDD/kg on the 5th, 10th, or 16th day of gestation, fetal liver PNP- and N-glucuronyltransferase and benzyrene hydroxylase activities were unaffected. However, after birth, marked increases over controls (3-10 fold) were observed. The inductive effect was caused both by newborn exposure to TCDD via maternal milk and the activation of an inducing mechanism after birth as evidenced by foster mother experiments. Glucuronidation rates of T, E₂, and DES were essentially unaffected in prenatal or postnatal animals. In addition to the above differences, T-, E₂-, and DES- (steroidal substrates) glucuronidations were not evident in the uterus, ovary, testes, and kidney whereas these tissues had high levels of non-steroid (PNP, N, MUB) GT. These data provide strong evidence for the multiplicity of GT.

The placental transfer rates of cadmium increased with increasing dose and increasing gestational age (12-20 days) after cadmium injection. Placental sufficiency tests and histological evaluations indicated that increased placental transfer was not related to placental damage. Preliminary evidence

suggests that effects on cadmium transfer results from saturation of metallothionein inducibility capacity at the higher doses.

Techniques have been developed to measure binding of steroids to cytoplasmic and nuclear receptors in several different tissues.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Perinatal development studies of drug-metabolizing enzymes have in the past dealt primarily with oxidative systems. However, the conjugative systems, especially, glucuronidation, are in many cases more important in fetal detoxication and steroid regulation pathways than are the mixed function oxidases. The few developmental studies that were done on conjugative systems were incomplete; dealing with only one enzyme in one tissue at one age and often using unsuitable incubation conditions. These studies will yield information concerning:

- (1) which organ or organs are most important in conjugation at different ages;
- (2) the type of fetal conjugate that is likely to be formed at particular times during the gestational period;
- (3) how the fetus independently detoxifies foreign compounds;
- (4) the role of conjugation pathways in the regulation of many endogenous compounds; and
- (5) the mechanisms responsible for developmental onset of GT.

Considerable research is in progress regarding the characterization of steroid receptors. However, relatively little attention is accorded the role that receptors play in developmental and reproductive toxicity. Our studies will attempt to characterize receptors as "markers" for certain types of carcinogenesis and teratogenesis.

Cadmium has been shown to be fetotoxic and teratogenic. One of the aims of our studies is to determine how the fetus and placenta detoxify cadmium and what role metallothionein inducibility plays in fetal detoxication pathways and placental transfer of cadmium and other heavy metals.

PROPOSED COURSE: We plan to concentrate our efforts for FY 76 on studying steroid metabolism and binding in target tissues with emphasis on effects of hormonally-active xenobiotics. The studies will include the characterization of cytoplasmic and nuclear receptors in developing tissues of normal and abnormal animals. This research effort will require the development of micro-assay techniques. Placental transfer studies of cadmium will continue with emphasis on the possibility that prior induction of maternal, fetal, or placental metallothionein might protect against fetotoxic effects of heavy metals. Studies of conjugative enzymes will attempt to discover the biochemical indicators for the developmental onset of activity.

KEYWORD DESCRIPTORS: metabolism; binding; steroids; xenobiotics; glucuronyltransferase; perinatal development; steroid receptors; developmental and reproductive toxicity; steroid metabolism; placental transfer; fetal binding; teratogenicity; microsomes; estradiol; diethylstilbestrol; uterus; vagina

PUBLICATIONS

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Project No.: Z01 ES 70140-03 ETB
1. Environmental Toxicology Branch
2.
3. Research Triangle Park, N.C.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

PROJECT TITLE: "Factors Affecting Hepatic Heme Synthesis During Mammalian Development"

PREVIOUS SERIAL NUMBER: NIEHS-ET-011

PRINCIPAL INVESTIGATOR: J. S. Woods, Ph.D.

OTHER INVESTIGATOR: None

COOPERATING UNIT: None

MAN YEARS

Total:	2.0
Professional:	1.0
Other:	1.0

PROJECT DESCRIPTION

OBJECTIVES: (1) To elucidate the mechanisms governing the regulation of hepatic hematopoiesis during mammalian development, (2) to investigate the mechanisms of alterations in the regulation of δ -aminolevulinic acid (ALA) synthetase during hepatocarcinogenesis induced by exogenous agents, (3) to characterize and study the effects of environmental agents on purified preparations of ALA synthetase from adult, fetal and neoplastic rat liver, (4) to determine the correlation between biochemical abnormalities associated with porphyrin overproduction and the clinical symptoms of porphyria, and (5) to provide a pharmacological and biochemical basis for predicting human responses to environmental agents which interfere with hepatic heme biosynthesis.

METHODS EMPLOYED: Standard published biochemical techniques are used to prepare subcellular fractions from mammalian tissues, to assay enzyme activities, and to determine porphyrin, heme precursor and protein concentrations. Techniques employed for the partial purification of ALA synthetase from rat liver mitochondria include solubilization with detergents, $(\text{NH}_4)_2\text{SO}_4$ fractionation, gel filtration, affinity chromatography, and sucrose density gradient centrifugation. Fetal animals of gestational age confirmed by date-breeding are used in developmental studies. Rats fed a diet containing a prescribed concentration of hepatocarcinogen for various time periods are utilized for studies of enzyme regulation and purification in/from precancerous and neoplastic liver.

MAJOR FINDINGS: Studies have been published on the role of heme in the regulation of ALA synthetase in fetal rat liver and on the partial purification and characterization of fetal ALA synthetase. These studies indicate that, in the fetus, ALA synthetase, the rate-limiting enzyme in heme biosynthesis in adults, is refractory to regulation by the mechanisms through which heme controls the activity of the adult enzyme. In addition, they suggest that fetal ALA synthetase may not become rate-limiting in hepatic heme biosynthesis until it becomes susceptible to repression by heme, a phenomenon which does not occur until shortly after birth. On the other hand, studies on the ontogenic development of other major enzymes of the heme biosynthetic pathway show that the activities of ALA dehydratase, uroporphyrinogen I synthetase and heme synthetase, and the affinities of these enzymes for their respective substrates, are all significantly greater than the activity or substrate (glycine) affinity of ALA synthetase throughout perinatal development. It is thus unlikely that enzymes other than ALA synthetase are present in rate-limiting states in fetal liver. Finally, studies on the partially purified preparation of fetal ALA synthetase indicate that this enzyme is significantly different from that of the adult in terms of its enhanced sensitivity to fluctuations in physiological parameters (pH, temperature, ionic strength) which characterize the rapidly changing fetal hepatocellular environment. Thus, the role of ALA synthetase as rate-limiting enzyme in fetal heme biosynthesis may be determined by gestational factors which include substrate availability and the physiological composition of the fetal liver cell, rather than by regulation by heme.

Studies currently in progress on the regulation of ALA synthetase in livers of rats fed the hepatocarcinogen, ethionine, suggest that ALA synthetase becomes refractory to repression by heme very early in the carcinogenic process. Subsequently, ALA synthetase becomes refractory to induction by agents which induce the enzyme in normal adult liver. This sequential loss of regulatory events suggest that these studies may be of value in identifying the order of physiological changes which occur in the selection of new cell populations during carcinogenesis. The susceptibility of ALA synthetase to both induction and repression in normal liver, but only to induction in the early stages of hepatocarcinoma, provides a biochemical basis for distinguishing between normal and premalignant tissues which may have diagnostic value in man.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: A knowledge of the mechanisms by which hepatic ALA synthetase, the rate-limiting enzyme in the heme biosynthetic pathway, is regulated at various stages of mammalian development is necessary to provide a clearer understanding of the means by which drugs and other environmental agents exert their effects on hematopoiesis, drug metabolism and cellular energy process in adult and perinatal animals. Identification of alterations in these regulatory mechanisms seen in precancerous and other pathologically altered tissues may enhance our understanding of carcinogenic processes and the mechanisms by which environmental agents induce malignant and other toxic changes in man.

PROPOSED COURSE: Studies concerning the regulation of hepatic hematopoiesis during mammalian development will continue. Particular emphasis will be placed on the investigation of the mechanisms by which ethionine and other carcinogens alter the regulation of ALA synthetase in adult rat liver. Further studies on the characterization of ALA synthetase partially purified from fetal and

malignant rat liver will be pursued with the aim of identifying the factors affecting the regulation of these enzymes in fetal and cancerous tissues. Studies designed to investigate the mechanisms by which prolonged exposure to low levels of selected environmental agents, such as heavy metals, affect the regulation of ALA synthetase and other mitochondrial enzymes will be initiated with the objective of providing a basis for the early prediction of hepatotoxicity in humans.

KEYWORD DESCRIPTORS: hepatic hematopoiesis; mammalian development; δ -aminolevulinic acid (ALA) synthetase; hepatocarcinogenesis; porphyria; heme precursor; hepatocarcinogen; enzyme regulation; precancerous and neoplastic liver; rate-limiting enzyme; refractory; ontogenic development; ALA dehydratase; uroporphyrinogen I synthetase; heme synthetase; perinatal development; hepatocellular environment; gestational factors; substrate availability; ethionine; selection of new cell populations, carcinogenesis; premalignant; hepatopoiesis; drug metabolism and cellular energy process; prediction; hepatotoxicity; repression; induction

PUBLICATIONS

Woods, J.S.: Studies on the role of heme in the regulation of δ -aminolevulinic acid synthetase during fetal hepatic development. Molecular Pharmacology, 10: 389-397, 1974.

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Woods, J.S. and Murthy, V.V.: δ -Aminolevulinic acid synthetase from fetal rat liver: Studies on the partially purified enzyme. Molecular Pharmacology, 11: 70-78, 1975.

Project No.: Z01 ES 70160-02 ETB
1. Environmental Toxicology Branch
2.
3. Research Triangle Park, N.C.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

PROJECT TITLE: "Developmental Toxicity of Medroxyprogesterone Acetate"

PREVIOUS SERIAL NUMBER: NIEHS-ET-003

PRINCIPAL INVESTIGATOR: F. D. Andrew, Ph.D.

OTHER INVESTIGATORS: P. E. Watkins, B.S., M.T.(ASCP) and R. L. Raynor, B.S.

COOPERATING UNIT: Research Triangle Institute (Procurement Contract: NIH-74C-390)

MAN YEARS:

Total:	1.8
Professional:	0.9
Other:	0.9

PROJECT DESCRIPTION

OBJECTIVES: (1) To determine whether administration of medroxyprogesterone acetate (MPA, Provera®) to pregnant animals interferes with development in utero. (2) To achieve a better understanding of the etiology of chemically-induced altered development.

METHODS EMPLOYED: MPA and related steroids were administered to pregnant mice, rats, and rabbits s.c. for various times during major organogenesis. Dosage levels tested ranged from the maximum tolerated dose for the dams to the apparent "no-effect" dose for the conceptus. Resulting offspring were examined for variations in the incidence of external, visceral, and skeletal alterations shortly before term. Maternal and fetal body weights and various maternal organ weights were measured to determine effects of steroid administration.

MAJOR FINDINGS: This project was first described (FY 1974) as part of efforts of Serial No. NIEHS-ET-003. Dose-related embryotoxicity was not observed in rats and mice at the highest dose and longest exposure tested (3000 mg/kg/day for 9 days). In contrast, embryoletality, stunting, and cleft palates were seen among the offspring of rabbits given 3, 10, or 30 mg MPA/kg/day for 3 days. The high cleft palate frequencies seen after MPA were unusual for rabbits since palate malformations rarely occur spontaneously and have seldom been induced experimentally except by glucocorticoids.

Changes in maternal organ weights of rabbits included hepatic hypertrophy plus adrenal and thymic involution; results consistent with glucocorticoid-induced effects on target tissues. When analogs of MPA and related steroids were tested in pregnant rabbits, only triancinalone acetate, a potent glucocorticoid, had a similar spectrum of effects to those of MPA. These results, and the fact that MPA has corticoid-like activity in rats and humans, suggest that MPA is teratogenic in rabbits because it has corticoid-like activity in this species also. An enigma in these results is the failure of MPA to induce corticoid-related malformations in susceptible mouse strains.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: A major impetus to evaluate MPA's embryotoxic potential in animals was a report by the Alert Practitioners Program that MPA and related steroids might be teratogenic in humans. Through the efforts described, MPA was evaluated in three species and an unusual species difference in response was revealed. The knowledge gained from the determination of the etiology of embryotoxicity and resolution of unusual species differences can improve our ability to predict fetal toxicity and/or teratogenicity.

PROPOSED COURSE: Most physiological effects induced by steroids in target tissues are initiated by interaction with specific cytoplasmic receptor proteins. MPA has not been tested previously for its binding to glucocorticoid receptors, but it has been shown to interact with uterine progesterone receptors of several species. MPA may interfere with development in utero by its effects on uterine accommodation, directly on the conceptus or indirectly by altering maternal endocrine and/or metabolic processes. To determine MPA's mode of action, its ability to interact with corticosteroid and progesterone receptors will be tested in target tissues of responsive and non-responsive species.

In anticipation of completing this project during the coming year, a feasibility study is currently underway to identify enzymes with important developmental and/or regulatory characteristics. Changes in the predictable patterns of enzyme differentiation during perinatal development may serve as sensitive and reliable indicators of dysfunction following prenatal chemical insult.

KEYWORD DESCRIPTORS: medroxyprogesterone acetate; pregnant animals; development; embryoletality, cleft palates, rabbits; glucocorticoids; cytoplasmic receptor proteins

Project No.: Z01 ES 70180-03 ETB
1. Environmental Toxicology Branch
2.
3. Research Triangle Park, N.C.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

PROJECT TITLE: "The Role of Superoxide Dismutase in Oxygen Tolerance and Oxygen Toxicity"

PREVIOUS SERIAL NUMBER: NIEHS-ET-012

PRINCIPAL INVESTIGATOR: J. D. Crapo, M.D.

OTHER INVESTIGATOR: None

COOPERATING UNIT: None

MAN YEARS

Total:	1.0
Professional:	1.0
Other:	0.0

PROJECT DESCRIPTION

OBJECTIVES: (1) To study the relationship between the level of superoxide dismutase (SOD) present in lung tissue and the ability of the animal to become "tolerant", i.e.: survive in pure oxygen or lethal doses of other pulmonary oxidants and (2) to determine the histological changes occurring in lungs exposed to oxidants and to identify the specific sites and characteristics of biochemical and physiological adaptation in "tolerant" animals.

METHODS EMPLOYED: The aerotoxicology facilities at the Institute are used to expose test animals to various concentrations of oxygen and aerosols of superoxide dismutase. Routine biochemical, histological, immunological, and physiologic testing procedures are also used. Pulmonary ultrastructure is characterized according to the morphometric procedures described by Weibel.

MAJOR FINDINGS:

(1) Pulmonary SOD and oxygen toxicity. Rats exposed to 85% oxygen for 7 days developed "tolerance" (the ability to survive in 100% oxygen) and at the same time the activity of pulmonary SOD increased 46% ($p < 0.01$). The rate at which rats exposed to 85% oxygen acquired "tolerance" paralleled the time course for increased SOD activity. The rate at which "tolerance" was lost when these rats were returned to air correlated closely with the decline of SOD activity to normal levels. Guinea pigs, hamsters, and mice were also studied. When exposed to 85% oxygen, these species failed to demonstrate higher activity of pulmonary

SOD and also failed to acquire "tolerance". These findings are consistent with the postulate that the increased tolerance to hyperoxia observed in rats is due to protection by SOD from the toxic effects of O_2^- .

(2) Characteristics of rat SOD. Two structurally different forms of SOD exist--the mitochondrial and the cytosol forms. Rat liver cytosol SOD was isolated, purified, characterized, and specific activity determined.

(3) Antibody titrations of pulmonary SOD. The increase in lung SOD activity of oxygen-exposed rats could be due to increases in either one or both forms of the enzyme. Also, the change in SOD activity could be due to increased amounts of the enzyme or to a change in enzyme properties. To study these questions, rabbit antisera specific for rat cytosol SOD was prepared and antibody titrations were performed on lung homogenates using agarose gels stained for SOD with nitroblue tetrazolium. SOD/lung increased 42% ($p < 0.01$) during hyperoxic exposure. Therefore, continuous exposure of rats to 85% oxygen led to increased absolute amounts of this enzyme rather than altering enzyme properties. The entire increase in SOD determined by activity assays (46%) could be accounted for by the increased amounts of cytosol SOD.

(4) Histological studies. Since the lung is a heterogeneous organ, the changes identified in SOD could be occurring in only a small portion of the many cell types. To identify the possible histological sites of the identified biochemical adaptation of oxygen, rat lungs were studied by light and electron microscopy and all major changes were quantitated by the morphometrical techniques described by Weibel. Alveolar type II cells and fibroblasts were found to proliferate and alveolar type II cell may play an important role in the adaptive responses of the lung, but further studies are needed to identify this specific cell type as the one becoming rich in SOD.

(5) Cross tolerance between NO_2 and O_2 . Animals made "tolerant" to O_2 were found to be partially cross tolerant to lethal doses of NO_2 , whereas animals made "tolerant" to NO_2 demonstrated only minimal cross tolerance to O_2 . The patterns of biochemical adaptation were similar in each case, but were more marked with exposures to oxygen and included adaptive changes in SOD, G6PD, catalase, peroxidase, lysosomal enzymes, and glutathione. The primary histological sites of damage were identified in each case and a theory developed to explain the different biochemical, histological, and physiological responses to different oxidants.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The single electron reduction of oxygen, producing an extremely toxic free radical known as superoxide, has recently been shown to be a biologically important reaction. The enzyme SOD dismutates this free radical directly to O_2 and H_2O_2 . It has been postulated that the presence of this enzyme protects against the toxic effects of oxygen and that it is necessary for survival in an oxygen-rich atmosphere. Oxygen is one of the most frequently used forms of medical therapy, and artificial atmospheres of oxygen are used in both industry and in medicine. All of the initial toxic effects of oxygen are manifest in the lung and deaths due to pulmonary oxygen toxicity are frequently reported. The current studies

represent one of the first attempts to propose a biochemical mechanism for pulmonary oxygen toxicity.

A large number of other environmental toxicants including ozone, NO_2 , and paraquat may produce part of their toxic effects by way of the superoxide free radical, and the current studies with SOD and oxygen may have broad applications with regard to toxicity of environmental agents.

PROPOSED COURSE: (1) The mitochondrial SOD which is being purified from rat liver will be characterized and used to produce a rabbit antisera specific for it. (2) Further antibody titrations using both cytosol SOD antisera and mitochondrial SOD antisera will further characterize changes in SOD during O_2 exposure. (3) Fluorescent antibody techniques will be used to determine the histological location of SOD in rat lung and ferritin-tagged antibodies will be used to further determine the subcellular distribution of the SOD enzymes. (4) Rats will be exposed to aerosols of SOD to attempt to modify the toxic effects of oxygen and Cu-deficient rats will be used to evaluate their expected increased sensitivity to oxygen. (5) Ozone exposed animals will be evaluated for the development of cross tolerance to oxygen exposure and for their ability to increase SOD activity in the lung.

KEYWORD DESCRIPTORS: superoxide dismutase; oxygen toxicity; tolerance; antibody titrations; morphometrical techniques; cross tolerance

HONORS AND AWARDS: November 1974 - Cecile Lehman Mayer Research Award, The American College of Chest Physicians

PUBLICATIONS

Crapo, J. and Tierney, D.: Superoxide dismutase and pulmonary oxygen toxicity. Am. J. Physiol. 226: 1401-1407, 1974.

Crapo, J.: Superoxide dismutase and tolerance to pulmonary oxygen toxicity. Chest. 62: 395-405, 1975.

Project No.: Z01 ES 70200-02 ETB
1. Environmental Toxicology Branch
2.
3. Research Triangle Park, N.C.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

PROJECT TITLE: "The Morphologic and Biochemical Effects of Toxic Metals"

PREVIOUS SERIAL NUMBER: NIEHS-ET-018

PRINCIPAL INVESTIGATOR: B. A. Fowler, Ph.D.

OTHER INVESTIGATORS: R. A. Goyer, M.D., Department of Pathology,
University of Western Ontario, London Ontario, Canada,
K. R. Mahaffey, Ph.D., F.D.A., Washington, D.C.
J. S. Woods, Ph.D., Environmental Toxicology Branch
G. W. Lucier, Ph.D., Environmental Toxicology Branch
G. E. R. Hook, Ph.D., Pharmacology Branch
M. R. Krigman, M.D., Department of Pathology,
University of North Carolina at Chapel Hill
P. Mushak, Ph.D., Department of Pathology,
University of North Carolina at Chapel Hill
D. A. Wolfe, Ph.D., Atlantic Estuarine Center,
National Marine Fisheries Service, Beaufort, N.C.
G. T. Barthalmus, Ph.D., Department of Zoology,
North Carolina State University at Raleigh
E. J. Faeder, Ph.D., EPA, NERC,
Research Triangle Park, N. C.
G. F. Nordberg, M.D., Department of Environmental Hygiene,
Karolinska Institute, Stockholm, Sweden

COOPERATING UNITS: Department of Pathology, University of North Carolina
at Chapel Hill
F.D.A., Washington, D.C.
Pharmacology Branch
National Marine Fisheries Service, Beaufort, N.C.
Department of Zoology, North Carolina State University
at Raleigh
Department of Environmental Hygiene, Karolinska Institute,
Stockholm, Sweden

MAN YEARS

Total:	3.6
Professional:	2.6
Other:	1.0

PROJECT DESCRIPTION

OBJECTIVES: (1) To define the ultrastructural and biochemical effects of toxic metals and other environmental toxicants on mammalian and marine invertebrate tissues, and (2) to correlate these observations with tissue levels and physiological alterations in blood and viscera.

METHODS EMPLOYED: Transmission electron microscopy, X-ray microanalysis, biochemical assay of microsomal enzymes and mitochondrial respiration, and routine blood chemistries were used.

MAJOR FINDINGS:

1. Arsenic. Combined oxygen electrode and ultrastructural studies were performed on kidneys of rats exposed to 40, 85, and 125 ppm levels of arsenate (As^{+5}) in the drinking water for 6 weeks. Decreased mitochondrial state 3 respiration was observed in all dose groups. Respiratory controls ratios were significantly lowered only in kidneys of rats given the 85 and 125 ppm dose levels. A dose related increase in swollen mitochondria and dense autophagic lysosomal bodies was confined to proximal tubule cells of these same animals.
2. Bismuth. Male rats were given intramuscular injections of a 50% solution of bismuth subnitrate (2 ml/kg body weight per day) for 4 days. Treated and control rats were killed at 2, 4, or 6 days after the last injection. Characteristic bismuth-induced nuclear inclusion bodies in renal proximal tubule cells were studied by X-ray microanalysis at 40, 60, and 80 keV accelerating voltages for bismuth. Optimal bismuth M_{α} and L_{α} X-rays peak to background ratios (P/B) were observed at the 40 keV accelerating voltage while the best analytical sensitivity $[(P/B) \times 10^{-3}]$ was recorded at the 80 keV voltage. This study provides basic information about the impact of accelerating voltage on X-ray microanalysis of heavy metals.
3. Cadmium. Ultrastructural studies were conducted on kidneys of rabbits given a single injection of cadmium-metallothionein at dose levels of 0.1, 0.2, and 0.4 mg Cd/kg body weight. The animals were killed 7 days after treatment. By light microscopy necrosis of proximal tubule cells was observed with the 0.4 mg Cd/kg dose level. At lower dose levels, increased numbers of swollen mitochondria and dense granular lysosomal bodies were noted in proximal tubule cells by electron microscopy. Oysters and clams were exposed to cadmium in sea water at a concentration of 0.1 ppm for 4 weeks. Approximately 3.5 pounds of cleaned shellfish tissues from these animals were frozen for subsequent incorporation into rat diets.
4. Methylmercury. Female rats were given access to drinking water containing methylmercury hydroxide (MMH) at concentrations of 3, 5, or 10 ppm MMH for 4 weeks prior to impregnation by non-treated males. Fetal and maternal liver mitochondria were studied for changes in ultrastructure, respiratory capacity, and protein synthesis on day 10 of pregnancy. Dose-related mitochondrial swelling associated with decreased state 3 respiration, respiratory control ratios, and protein synthesis was observed in fetal and to a lesser degree in maternal mitochondria.

5. Dioxin. Previous studies had shown marked induction of renal microsomal enzyme activities associated with proliferation of smooth endoplasmic reticulum (SER) in pars recta (S_3) proximal tubule cells of dioxin treated rats. Male rats were killed 6 days after a single oral dose of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) at a dose level of 25 $\mu\text{g/kg}$ and the kidneys dissected into medulla, outer stripe of the medulla, and cortex for assay of benzpyrene hydroxylase and glucuronyltransferase activities. The largest increase in these enzyme activities occurred in the outer strip of the medulla which has the highest concentration of pars recta (S_3) cells.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: These studies attempt to delineate ultrastructural and biochemical criteria for evaluating the toxicity of environmental agents administered at low levels. These findings are associated with physiological changes and tissue concentrations of these agents. The studies of marine organisms are of value because man is commonly exposed to heavy metals through consumption of shellfish. A knowledge of where and how these metals are localized within mammals following the route of exposure is of great potential value.

PROPOSED COURSE: The effects of heavy metals on cellular ultrastructure will be continued and much greater emphasis will be placed upon correlation with biochemical alterations in affected organelles. In addition, the influence of one heavy metal on the toxicity of another will also be investigated. Further studies are anticipated in order to compare observed cellular alterations of heavy metals injected into tissues with those seen when the metals are given in drinking water or added to food. More extensive comparative marine studies are also anticipated.

KEYWORD DESCRIPTORS: arsenic/kidney; decreased state 3 respiration and respiratory control ratios; cadmium kidney necrosis of proximal tubule cells; swollen mitochondria; dense granular lysosomal bodies; Bi inclusions X-ray microanalysis M_α and L_α X-ray peaks; methylmercury/maternal and fetal liver; TCDD/induction of kidney microsomal enzymes/benzpyrene hydroxylase, glucuronyltransferase/outer strip of medulla

PUBLICATIONS

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Fowler, B.A. and Parker, P.: Observations of the preparation of epoxy embedded tissue samples for energy-dispersive X-ray analysis. Stain Technol. 48: 333-335, 1973.

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Fowler, B.A. and Weissberg, J.B.: Arsine poisoning. New Eng. J. Med. 291: 1171-1174, 1974.

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Fowler, B.A.: Lead levels in dover sole collected in southern California coastal waters. Proceedings National Conference on Health Effects of Occupational Lead and Arsenic Exposure. (in press).

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Project No.: Z01 ES 70220-02 ETB
1. Environmental Toxicology Branch
2.
3. Research Triangle Park, N.C.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

PROJECT TITLE: "Interaction of Complexing Agents with Heavy Metals and Effects on Teratogenicity"

PREVIOUS SERIAL NUMBER: NIEHS-ET-005

PRINCIPAL INVESTIGATOR: C. A. Kimmel, Ph.D.

OTHER INVESTIGATORS: C. D. Sloan, M.S. and S. Gipson, B.S.

COOPERATING UNIT: None

MAN YEARS

Total: 2.00

Professional: 0.85

Other: 1.15

PROJECT DESCRIPTION

OBJECTIVE: To investigate metal chelation as a mechanism of teratogenic action by studying the interaction of EDTA with essential metals.

METHODS EMPLOYED: Animals were maintained on purified diets during pregnancy to eliminate the variability of heavy metal contamination in commercial feed. EDTA was administered in the diet and this treatment group was compared with animals given a zinc-deficient diet. Changes noted in the gonads of offspring were studied histologically in order to compare the specific effects of EDTA and zinc-deficiency on gonadal development. A few animals treated with EDTA were allowed to litter, and offspring were compared with controls in terms of survival to weaning and growth rate. The gonads were examined microscopically at various times after weaning.

MAJOR FINDINGS: EDTA treatment and zinc-deficiency produce very similar types of malformations in rats. Gonad dysgenesis was seen in many malformed fetuses so that it was impossible to determine the sex of some of these on gross observation of the gonads. Histologic sections of gonads from EDTA-treated and zinc-deficient fetuses were almost identical in appearance. The organ size and number of cells, particularly germ cells, was greatly reduced in both ovary and testis. These data support the hypothesis that EDTA alters development primarily by interfering with zinc.

Postnatal development of offspring of EDTA-treated animals was severely affected. Survival of pups to weaning was greatly reduced, and growth rate (especially for males) was significantly lower than that of controls. Time of vaginal opening did not appear to be altered in female pups, and no striking changes have been noted in the preliminary histologic observation of gonads. However, since those animals with altered gonadal development were usually seriously malformed, it is probable that only the more normal offspring survived to weaning.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Many chelating agents are found in the environment (i.e., as natural products, food preservatives, and pollutants) and may have significant effects in areas where essential metal deficiencies occur. The interaction of such chelators with essential metals could produce a temporary metal deficiency and result in inhibition of enzymes necessary for critical stages of development. This project is designed to develop methods of studying environmental compounds for chelating ability using EDTA as a model compound.

PROPOSED COURSE: Future studies will focus on behavioral alterations in offspring of animals administered nutritional imbalances or toxic heavy metals during pregnancy.

KEYWORD DESCRIPTORS: metal chelation; mechanism of teratogenic action; interaction; essential metals; zinc-deficient; gonad dysgenesis; postnatal development; EDTA

Project No.: Z01 ES 70240-02 ETB

1. Environmental Toxicology Branch
- 2.
3. Research Triangle Park, N.C.

PHS-NIH

Individual Project Report

July 1, 1974 through June 30, 1975

PROJECT TITLE: "The Mode of Toxicity of Organochlorine Xenobiotics
in Cultured Mouse Lymphoma Cells"

PREVIOUS SERIAL NUMBER: NIEHS-ET-017

PRINCIPAL INVESTIGATOR: J. W. Spalding, Ph.D.

OTHER INVESTIGATOR: J. Z. Gabliks, Ph.D., Visiting Scientist

COOPERATING UNIT: None

MAN YEARS

Total:	1.5
Professional:	1.0
Other:	0.5

PROJECT DESCRIPTION

OBJECTIVES: To study the nature of the interaction of organochlorine compounds with the plasma membrane of mammalian cells grown *in vitro*, and to determine how this interaction relates to the toxic properties of these same organochlorine compounds.

METHODS EMPLOYED: The effect of the interaction of organochlorine compounds with the plasma membrane of mouse L5178Y lymphoma cells is observed in susceptible and resistant lymphoma cell sublines. The parameters studied are: (1) the transport into cells and subsequent incorporation of appropriate precursors into DNA, RNA, protein, and phospholipids and (2) the degree of binding which occurs between the organochlorine compounds and the cell plasma membrane.

MAJOR FINDINGS:

(1) Representatives of several different classes of organochlorine compounds, e.g., DDT, chlordane and 4-chlorobiphenyl (4-Cl-BP), are toxic to L5178Y cells. 4-Chlorobiphenyl inhibits the rate of transport of selected small molecular weight precursors of protein and nucleic acid syntheses. All three above named organochlorine compounds stimulate the incorporation of choline into phosphatidylcholine but have little or no effect on the rate of choline transport across the cell membrane. In contrast to the stimulatory effect on phosphatidylcholine (PC) synthesis, 4-Cl-BP inhibited the incorporation of ethanolamine

into phosphatidylethanolamine (PE) under similar cultural conditions.

(2) Clones of L5178Y mouse lymphoma cell sublines have been isolated, which have the ability to grow in culture medium containing a greatly reduced serum content, e.g., up to 100-fold less serum. PC synthesis in these sublines is stimulated by 4-Cl-BP to the same degree as in the parent cell line. However, several of these sublines synthesize PC at a much higher rate. Of the choline taken up by the cells, 25-30% is incorporated into PC after one hour of incubation in the presence of 4-Cl-BP. In addition, these sublines are more sensitive to organochlorine toxicity. This increase in magnitude of response to 4-Cl-BP provides a better opportunity to study the mechanism of organochlorine toxicity.

(3) The effect of 4-Cl-BP on ^{14}C -methylcholine incorporation into the different membrane components of the cell has been investigated. The stimulation of PC synthesis occurred to a similar degree in the membrane components of the nuclear, mitochondrial and microsomal fractions of the cell. Thus, it was concluded that the effect of 4-Cl-BP on PC synthesis was not specific for any particular membrane-containing organelle of the cell. These results imply that 4-Cl-BP increases the rate of enzyme activity of those enzymes concerned with de novo synthesis of PC.

(4) The effect of organochlorine agents on several stages of the cell replication cycle has been investigated. DDT and 4-Cl-BP do not inhibit cytokinesis in cells rescued from a block in DNA synthesis (S-phase) subsequent cytokinesis is inhibited. These results suggest that these agents are preventing cells from completing late S-phase, or the G_2 -phase of the cell cycle.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The mode of toxicity exhibited by the organochlorine pesticides and other related xenobiotics, e.g., PCB's, is still unknown at this time. Tissue culture cell systems provide useful models whereby the molecular mechanism of toxicity expressed by these compounds may be investigated. The studies described may lead to an understanding of how DDT, chlordane, PCB's, and other highly water-insoluble and persistent organochlorine compounds effect their toxicity in biological systems.

The results of the investigations described above suggest that the organochlorine compounds express their toxicity by altering the plasma membrane in such a way that the rate of transport of essential small precursor molecules into cells (tissues) is effected. In addition, they alter the metabolism of phospholipids and thus, can effect the integrity of the various membrane structures of cells (tissues).

PROPOSED COURSE:

(1) The effect of organochlorine agents on phospholipid metabolism and transport among the different membrane-containing organelles of the cell will continue to be defined.

(2) The nature of the interaction between organochlorine compounds and the plasma membrane of cells will continue to be investigated.

(3) The de novo synthetic pathway of phosphatidylcholine synthesis is comprised of three intermediate steps. The enzymatic character of each step has been defined and enzyme activity may be studied by using the appropriate substrates. An attempt will be made to determine the effect of organochlorine agents on these enzymes in treated cells.

KEYWORD DESCRIPTORS: xenobiotics; organochlorine compounds; plasma membrane of mammalian; lymphoma cell sublines; phospholipids; protein and nucleic acid synthesis; de novo

PUBLICATIONS

Spalding, J.W., Ford, E., Lane, D., and Blois, M.: The character of 1,1,1-trichloro-2,2-bis (p-chlorophenyl) ethane resistance in mouse L5178Y lymphoma cells. Biochem. Pharmac. 20: 3185-3196, 1971.

Spalding, J.W., Ford, E., and Lane, D.: The effect of 4-chlorobiphenyl on substrate transport and phospholipid metabolism in mouse L5178Y lymphoma cells. Accepted by Biochemical Pharmacology, 1975.

Project No.: Z01 ES 70241-04 ETB
1. Environmental Toxicology Branch
2.
3. Research Triangle Park, N.C.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

PROJECT TITLE: "In Vitro Culturing of Mammalian Gonadal Tissues"

PREVIOUS SERIAL NUMBER: NIEHS-ET-016

PRINCIPAL INVESTIGATORS: J. W. Spalding, Ph.D., and J. A. McLachlan, Ph.D.

OTHER INVESTIGATORS: None

COOPERATING UNIT: None

MAN YEARS

Total:	1.2
Professional:	0.6
Other:	0.6

PROJECT DESCRIPTION

OBJECTIVE: To develop a viable tissue or organ culture system of mammalian gonadal tissue for the purpose of investigating the mechanism by which chemical agents affect oogenesis and the subsequent stages of oocyte maturation and follicular development and maturation.

METHODS EMPLOYED: Fetal gonads from C 57 B1/6 mouse are removed at various intervals during gestation and explanted as organ cultures in vitro. Ovaries at different stages of maturation are removed from prepubertal mice and explanted in organ culture in a similar manner. Tissue culture medium supplemented with fetal calf serum is used to support explant growth. The growth and differentiation of different cell types are followed by morphological and histochemical parameters.

MAJOR FINDINGS:

(1) Oocyte maturation in ovary explants taken from embryos at 16 days gestation time was followed for 12-14 days in culture. Oocyte maturation in vitro occurred in a manner analogous to oocyte maturation in vivo up to 25 days post-conception time. Follicle maturation proceeded to the bilaminar and trilaminar stage, but the rate of follicle maturation was retarded compared to that which occurred in vivo. Temperature variation and an alteration in medium-change schedules did not improve the rate of follicle maturation in vitro. Growth and development in the explants was similar at both 34 and 37°C. Tissue viability

and the rate of follicle maturation was not improved by changing the growth medium (refeeding) every day. Testosterone, estrogen, progesterone, FSH, and LH had no discernible effect on the progression of early oocyte and follicle maturation. It appears that the 16 day fetal mouse ovary is sufficiently programmed at this stage of fetal development so that it can remain unresponsive to a hormonal environment that will subsequently have profound effects in the post-pubertal period.

(2) The characterization of growth and maturation in explants of 12 day fetal ovaries is under continuing investigation. Oogonia continue to replicate for several days in culture. However, during this same interval, the proliferative activity of the stromal cells surrounding the oogonia and oocytes is low. Twelve day fetal ovaries appear to grow equally well at both 34 and 37°C. A daily refeeding schedule did not enhance growth nor did it prevent oocyte degeneration after 7 days in culture. Twelve day fetal ovaries, like the 16 day fetal explants, were unaffected by the steroid hormones and gonadotrophins cited above.

(3) Prepubertal ovaries have been excised from C 57 B1 mice at 5, 10, 15, and 22 days of age and cultured as explants. Follicle maturation continues in vitro, but the rate of maturation is retarded in comparison to the rate of maturation in vivo. It appears that several critical stages of follicle maturation cannot be attained in vitro under present culture conditions.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Various environmental agents, including drugs, are known to effect reproduction and development. The specific molecular effect of these agents on germ cells (oocytes and spermatocytes) during the early stages of development are relatively unknown. It can be expected that defective germ cells may result in offspring which are sterile or which have a wide range of inborn genetic deficiencies. It would be beneficial to have an in vitro model of embryonic and postnatal germ cell differentiation. In vitro studies could then be correlated with ongoing studies of the in vivo effects of chemicals on gametogenesis and fertility. Hopefully, a rapid in vitro test system can be developed to predict in vivo effects of chemicals. In addition, this in vitro system would provide an opportunity to further study mechanisms which regulate differentiation and maturation.

PROPOSED COURSE:

(1) In vitro studies with fetal and prepubertal ovarian explants will continue to be directed toward obtaining consistent and reproducible stages of growth and maturation. This test system will then be utilized to develop parameters that may be used to examine the effect of selected xenobiotics on oocyte growth and maturation.

(2) The factors which regulate early follicle growth and maturation in prepubertal mice are unknown. These growth factors may be produced by various cell types within the ovary itself, by extra-ovarian tissues, or depend on a complex interaction between both of the aforementioned; the latter case seems most likely. Therefore, it is of importance to define the stages of early follicle growth and maturation that can be achieved in vitro. Studies of this nature have first priority and are in progress.

(3) A model ovotoxin, 7,12-dimethylbenz (a) anthracene causes atresia, oocyte degeneration, and ultimately granulosa cell carcinoma in mice. The in vivo effects of this ovotoxin on early follicle development and maturation will be compared with its effects on ovarian explants in vitro.

KEYWORD DESCRIPTORS: oogenesis; oocyte maturation; follicular development; maturation; in vitro; differentiation; in vivo; xenobiotics

Project No.: Z01 ES 70260-03 ETB

1. Environmental Toxicology Branch

2.

3. Research Triangle Park, N.C.

PHS-NIH

Individual Project Report

July 1, 1974 through June 30, 1975

PROJECT TITLE: "Application of Bone Marrow Cell Culture and Cell Separation Techniques to the Study of the Hematologic Effects of Environmental Agents

PREVIOUS SERIAL NUMBER: NIETS-ET-013

PRINCIPAL INVESTIGATOR: J. B. Weissberg, M.D.

OTHER INVESTIGATORS: R. T. Drew, Ph.D., Pharmacology Branch and I. P. Lee, Ph.D.

COOPERATING UNIT: Aerotoxicology Section, Pharmacology Branch

MAN YEARS:

Total: 1.1

Professional: 1.1

Other: 0.0

PROJECT DESCRIPTION

OBJECTIVES: The aim of these studies has been the development of in vitro techniques for the investigation of the effects of environmental agents upon hematopoiesis. A tissue culture system that allows sustained proliferation and differentiation of bone marrow cells has been developed for the purpose of (1) identifying agents that are toxic to the marrow, (2) evaluating the role of metabolism in the action of various toxins, and (3) elucidating mechanisms of toxicity. The separation of bone marrow cells into morphologically homogeneous cell populations has been sought as an adjunct to the study of hematopoietic cells in culture. Successful isolation of individual cell types will facilitate the identification of cell-specific effects of environmental toxins and permit cell-cycle kinetic studies of bone marrow cells.

METHODS EMPLOYED: Rabbit bone marrow cells are cultured utilizing a liquid culture technique based on an in vitro diffusion chamber principle. Cells are maintained at high concentration within a stemmed glass bulb closed at one end by a dialysis membrane. The bulb is suspended in an Erlenmeyer flask fitted with an air vent for gas exchange and filled with tissue culture medium. Flasks are incubated at 37°C in 7.5% CO₂. Cells proliferate both in suspension in the inner medium and adherent to the surface of the dialysis membrane. Bone marrow cell separation is accomplished by the velocity sedimentation technique, by which cells of different size sediment at a differential rate through a medium

with a small density variation sufficient only to prevent disturbances as a result of convection currents.

MAJOR FINDINGS: The velocity sedimentation technique allows a clear separation of nucleated erythroid cells from cells in the granulocytic series. Among the granulocytic cells there appears a useful separation of blasts, metamyelocytes, myelocytes and mature neutrophils, but there is some overlap. Culture of bone marrow cells in the liquid system shows sustained cellular proliferation and differentiation. Conditions appear to favor granulopoiesis and macrophage morphogenesis. However, maturation along erythroid and lymphoid cell lines is also observed. In initial studies benzene has been employed as a model agent to investigate the applicability of in vitro techniques to the study of the hematologic effects of environmental toxins. When different concentrations of benzene are allowed to equilibrate with the culture medium, a dose-related effect on cellular proliferation is observed. A decline in viable cell numbers as well as an arrest in cellular maturation are observed at low benzene concentrations.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: In vitro methodology allows study not only of animal but also of human hematopoietic tissue. Agents that cannot be safely or easily administered to human subjects, the toxicity of which may or may not have been documented in animal studies, can be investigated in vitro with regard to their effects on human cells. Furthermore, the manipulations to which an in vitro system lend itself are ideal for the purpose of evaluating the mechanisms by which an agent is toxic to the bone marrow. Benzene, the model agent chosen for the present studies, has been implicated not only in aplastic anemia but also in leukemia. In vitro study of benzene and other such agents might elucidate mechanisms not only of bone marrow aplasia but also of malignant transformation.

PROPOSED COURSE: The culture of rabbit bone marrow cells separated into homogeneous populations by the velocity sedimentation technique is now being undertaken. Further studies planned include evaluation of the in vitro effect of benzene on individual cell populations. The effects of benzene on lymphoma cell cultures will be compared to those observed in the bone marrow cell culture system. Also planned are comparisons of the effects on rabbit bone marrow in vitro between benzene and its non-toxic alkyl derivatives and between benzene and some of its phenolic breakdown products. The combined effect of benzene and liver microsomes on bone marrow cultures will also be studied. The culture of bone marrow cells from rabbits exposed to benzene in vivo will be evaluated. And finally, the effects of benzene on human hematopoietic tissue will be compared to observations derived from animal studies.

KEYWORD DESCRIPTORS: bone marrow cell culture; cell separation techniques; in vitro techniques; hematopoiesis; bone marrow cells; liquid culture technique; velocity cell sedimentation; benzene

PUBLICATIONS

Weissberg, J.B. and Zinkl, J.C.: Effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin upon hemostosis and hematologic function in the rat. Environ. Health Persp. 5: 119-123, 1973.

Weissberg, J.B. and Voytek, P.E.: Liver and red cell porphobilinogen synthase in the adult and fetal guinea pig. Biochimica et Biophysica Acta. 364: 304-319, 1974.

Fowler, B.A. and Weissberg, J.B.: Arsine poisoning. New Eng. J. Med. 291: 1171-1174, 1974.

Project No.: Z01 ES 70280-02 ETB

1. Environmental Toxicology Branch
- 2.
3. Research Triangle, Park, N.C.

PHS-NIH

Individual Project Report

July 1, 1974 through June 30, 1975

PROJECT TITLE: "Role of Plasma Membranal Systems in Regulation of Behavior and Development of Fetal-Embryological Tissues"

PREVIOUS SERIAL NUMBER: NIEHS-ET-021

PRINCIPAL INVESTIGATOR: W. E. Wilson, Ph.D.

OTHER INVESTIGATORS: P. W. Albro, Ph.D., R. E. Staples, Ph.D. and
E. R. Soares, Ph.D.

COOPERATING UNITS: Environmental Biology and Chemistry Branch and
Environmental Mutagenesis Branch

MAN YEARS

Total:	2.40
Professional:	1.10
Other:	1.30

PROJECT DESCRIPTION

OBJECTIVES: (1) To ascertain the extent to which modulation and/or modification of plasma membranal structure and function correlates with fetal toxic and/or teratogenic responsiveness. (2) To characterize the properties and behavior of plasma membranal regulatory enzymes from fetal tissues and, where possible, to compare behavior of enzymes from fetal tissues with the corresponding ones from the adult. (3) To obtain further insight into the biochemical basis for the occurrence of abnormal ectodermal development (AED) about the brain and spinal cord.

METHODS EMPLOYED: Feeding studies require the use of completely defined diets, pair feeding, occasional diet modification and teratological examination. Behavior of fetal ($\text{Na}^+ + \text{K}^+$)ATPases and prostaglandin synthetases have been examined using conventional techniques for enzyme purification and characterization. Tissue lipid analyses were performed using thin-layer and gas chromatography coupled with various chemical analyses. Chemical syntheses were required for preparation of several analogs of essential nutrients.

MAJOR FINDINGS: Aspirin administration to pregnant rats elicits AED (exencephaly, spina bifida, exposure of the peritoneal cavity, etc.) in fetal rats, however, indomethacin does not elicit the same spectrum of responsiveness. The AED in response to aspirin is augmented by simultaneous administration of

a diet deficient in pantothenic acid (PA) and, possibly, essential fatty acids (EFAs).

Pregnant rats fed a diet deficient in PA, with or without EFAs, from the day of breeding produced fetuses whose gross appearance was normal. However, grossly observable AED occurred in fetal animals from pregnant rats which had been fed (a) a PA-deficient diet, during pregnancy, supplemented only on day-1 with a PA-antimetabolite or (b) a diet deficient in PA from several (3 to 12) days prior to breeding through pregnancy.

The relative content of linoleate and arachidonate in phospholipids from placental microsomes has been found to reflect the type of fatty acids in lipids fed the maternal animal during pregnancy. However, the arachidonate content of phospholipid from fetal brain was not influenced by the dietary lipids. This indicated that the arachidonate content of developing nerve cells is closely regulated. Such regulation may be a reflection of a requirement for arachidonate for maintenance of normal nerve membranal fluidity and/or for neuronal synthesis of prostaglandins.

The $(Na^{+}+K^{+})$ ATPase from fetal brain has been partially purified and its behavior has been compared with that of the enzyme from maternal brain.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: A variety of types of AED occur in humans. In experimental animals AED (exencephaly, spina bifida, etc.) can result from nutritional variations in diets fed pregnant animals as well as from exposure of pregnant animals to drugs, electromagnetic radiation and various environmentally significant chemicals. An understanding of the biochemical basis for such AED could permit anticipation of and/or protection against their causes.

These feeding studies can be interpreted to support the formulation of a hypothesis to the effect that altered prostaglandin biosynthesis may provide a plausible biochemical basis for rationalization of the above-mentioned types of AED. In view of the paucity of available information, an effort should be undertaken to ascertain whether it will be feasible to characterize the behavior of prostaglandin synthetases and/or nucleotide cyclases in embryonic mammalian cells and/or in uterine endometrial cells. An understanding of the behavior of the regulatory enzyme systems could be beneficial in predicting responsiveness to various environmental alterations.

PROPOSED COURSE: Feeding studies will involve determination of (a) teratogenicity of other drugs and agents which yield a high incidence of AED, (b) the influence of PA-deficiency on fetal responsiveness to other teratogens, (c) the teratogenicity of essential fatty acid (EFA) deficiency and/or of geometrical isomers of EFAs.

Biochemical studies will involve an effort to ascertain the roles of prostaglandins and/or cyclic nucleotides in regulating behavior and/or development of embryonic cells. This will initially involve examination of prostaglandin synthetases and/or nucleotide cyclases of uterine, placental and/or several fetal tissues. Where appropriate, properties of fetal enzymes will be compared with those of the adult.

As genetic factors seem to be related to elicitation of AED by administration of deficient diets to pregnant rats, secondary efforts will be undertaken to determine the feasibility of using cultured embryos and/or to obtain a sub-strain which will yield a high incidence of AED as a consequence of deficiencies of essential nutrients in maternal diets.

KEYWORD DESCRIPTORS: fetal; teratogenic; abnormal ectodermal development (AED); plasma membranal regulatory enzymes; (Na⁺+K⁺)ATPases and prostaglandin synthetases; exencephaly; spina bifida

Project No.: Z01 ES 70300-05 ETB

1. Environmental Toxicology Branch

2.

3. Research Triangle Park, N.C.

PHS-NIH

Individual Project Report

July 1, 1974 through June 30, 1975

PROJECT TITLE: "The Marsupial Neonate as a Model for the Identification and Evaluation of Environmental Toxicants"

PREVIOUS SERIAL NUMBER: NIEHS-ET-022

PRINCIPAL INVESTIGATOR: W. Jurgelsky, Jr., Ph.D., M.D.

OTHER INVESTIGATORS: P. M. Hudson, B.S.,
W. Wood, Ph.D., C. Dawe, M.D., M. Chirigos, Ph.D., and
J. Rice, Ph.D., NCI
A. Moore, P. Mortillaro, J. Romine and A. Godwin, MAPB
R. Martensen, Ph.D., NIMH
G. Alderson, D.D.S., M.D., C. Boyers, D.D.S.,
C. J. Davis, M.D., L. Zimmerman, M.D., K. Ishak, M.D.,
F. Mostofi, M.D., and L. Johnson, M.D., AFIP
U. J. Lewis, Ph.D., Scripps Institute

COOPERATING UNITS: Laboratory of Pathology, Viral Leukemia and Lymphoma
Branch, Experimental Pathology Branch, and Viral Oncology
Branch, NCI
Medical Arts and Photography Branch, NIH
Laboratory of Cerebral Metabolism, NIMH
Oral, Ophthalmologic, Orthopedic, Hepatic, Renal and
Pulmonary Branches, AFIP
Scripps Institute, LaJolla, California

MAN YEARS

Total:	4.5
Professional:	2.5
Other:	2.0

PROJECT DESCRIPTION

OBJECTIVES: (1) To develop techniques for efficient breeding of the opossum under laboratory conditions and (2) to characterize the neonatal and developing opossum anatomically and physiologically and to determine their pathophysiological responses to selected environmental toxics and carcinogens.

METHODS EMPLOYED: Special pharmacological techniques, light and electron microscopy, and standard biochemical methods of ultracentrifugation, thin-layer chromatography and gas chromatography are utilized.

MAJOR FINDINGS:

(1) Breeding and Laboratory Management of the Opossum. Three papers summarizing this work have been published (see below).

(2) Opossum Film. Completion of the films entitled: "The American Opossum as a Biomedical Model." Part I: "The Neonatal and Developing Opossum" and Part II: "The Adult Opossum" continues to be delayed due to unavailability of resources. NIEHS management has indicated that funds will not be released for the purpose of completing this work until the carcinogenesis work is complete. On this basis, the projected completion date for this project is Fall of 1977 or later.

(3) Biologic Characterization of the Opossum. (a) Studies to isolate and characterize growth hormone and prolactin from the opossum pituitary with Dr. U. J. Lewis, Scripps Institute, LaJolla, California, are being continued. The collaborator is in the process of analyzing pituitaries from 55 opossums. There are no results to report to date. (b) A study of morphology and myogenic potential of the skeletal muscle of the opossum with Dr. Brody, Department of Neurology, Duke University Medical Center has been aborted due to loss of resources. (c) Light and electron microscopic studies of developing opossum skeletal and cardiac muscle with Dr. J. Sommers, Department of Pathology, Duke University Medical Center have been aborted due to unavailability of newborn opossums. (d) Isolation and electrophoretic characterization of brain protein in the opossum with Dr. R. E. Martensen, Laboratory of Cerebral Metabolism, NIMH, are being continued. There are no results to report.

(4) Carcinogenesis Study. Work continues on the autopsy phase of the experiments in which 100 mg/kg of ethyl nitrosourea was orally administered to opossums at several stages of development. The carcinogen was given as a single dose or as four 25 mg/kg increments.

A further spectrum of epithelial and mesenchymal neoplasms have been identified in the treated animals including several types of embryonal tumors not previously inducible by the transplacental route in eutherian laboratory mammals. The embryonal neoplasms include five intraocular teratoid medulloepitheliomas (to our knowledge these neoplasms represent the first intraocular tumors of any type induced systemically), a high incidence of nephroblastomas (unlike so called nephroblastomas) induced in other species, the opossum tumors are morphologically identical to those in man in that they contain connective tissue, tubules, and pseudoglomeruli, hepatoblastomas, myomas of the jaw, gangliogliomas or tumors of the neuron (not previously reported in other species), primitive neuroectodermal tumors of the brain, ameloblastomas, and osteomas of the jaw. Associated with the neoplasms is a spectrum of teratologic changes including hypoplasia of the kidney, hypoplasia of the female genitalia, hepatic cysts, cysts of the kidney ranging from simple to multiloculated cysts to true polycystic kidneys, dysgenesis of the kidney, and hemangiomas of jaw, skin, liver and epididymis.

In a number of instances, neoplasms have been found to be present at the site of the malformations, an association again not previously reproduced experimentally.

In the FY 1974 annual report, we included hermaphroditism as one of the observed teratologic changes. Since that time, several additional cases of hermaphroditism have been identified (for a total of 10 cases) in the control population of opossums utilized in the cancer study. This leads to the conclusion that this malformation is either indigenous to the opossum or, less likely, that it is the result of exposure to some unknown factor in the environment of the captive opossum. While all animals have not been examined as yet, the incidence of hermaphroditism is already higher than that previously reported for other species.

Neither the autopsy phase of the pilot study to compare the effects of intraperitoneal and intraocular administration of ENU with the effects of oral administration nor the autopsies in the murine RNA virus study have as yet been completed.

The retrospective study to determine the state of organogenesis at the time of ENU induction of organ specific tumors has been delayed because of a lack of adequate numbers of animals for this work.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Our results to date imply that the opossum, early in postnatal life, may be a useful model system for the study of certain of the major dysontogenic neoplasms which have been difficult or impossible to reproduce in the standard laboratory species. The ability to induce embryonal tumors in the opossum may be explained in part by the unique opportunity in this species for the direct exposure of slowly maturing embryonic and fetal tissue to carcinogens in the absence of interference attributable in the eutherian placental mammal to the mother, the placenta and the fetoplacental unit. A more important factor facilitating the induction of such tumors in this species may be a slow sexual maturation and a relatively late postnatal initiation of endocrine and lymphoid function, possibly providing a longer period than in the eutherian laboratory mammal for the initiation and development of the oncogenic process in the absence of the inhibition often associated with full endocrine and immunologic function.

There appear to be a number of advantages of the marsupial model for embryonal carcinogenesis. They include an opportunity to explore the apparent relationship between oncogenesis, teratogenesis, and mutagenesis in the absence of the major handicap intrinsic to the eutherian animals, namely, the impossibility of distinguishing direct carcinogen induced teratologic or mutagenic changes from identical lesions which are the indirect result of adverse physical and physiologic effects of the carcinogen on the maternal or fetoplacental unit. In the semiembryonic, semifetal opossum orally or parenterally exposed to a carcinogen, mutations and malformations can only be either carcinogen induced or spontaneous.

PROPOSED COURSE:

(1) Carcinogenesis Study. Histologic preparation of tissues and histopathologic interpretation and classification of lesions will continue as resources allow. The current level of technical support and resources will greatly delay completion of this work for publication.

(2) Opossum Films. Completion of the films will depend on the availability of pregnant opossums and technical and fiscal support.

KEYWORD DESCRIPTORS: opossums; biomedical models; Ethyl nitrosourea; embryonal tumors; carcinogenesis

PUBLICATIONS

Jurgelsky, W.: The opossum (Didelphis virginiana Kerr) as a biomedical model. I. Research perspective, husbandry and laboratory techniques for the opossum. Lab. Animal Sci. 24: 375-403, 1974.

Jurgelsky, W., Forsythe, W., Dahl, D., Thomas, L.D., Moore, J.A., Kotin, P., Falk, H.L., and Vogel, F.S.: The opossum (Didelphis virginiana Kerr) as a biomedical model. II. Breeding the opossum in captivity: Facility design. Lab. Animal Sci. 24, 404-411, 1974.

Jurgelsky, W. and Porter, M.E.: The opossum (Didelphis virginiana Kerr) as a biomedical model. III. Breeding the opossum in captivity: Methods. Lab. Animal Sci. 24, 412-425, 1974.

PHARMACOLOGY BRANCH

PHARMACOLOGY BRANCH Summary Statement

The Pharmacology Branch has five or six program areas organized into four formal sections and one work group. The major emphasis of the research of the Branch is concerned with mechanisms by which environmental pollutants and effectors cause toxicity, the development of better test systems for detecting and evaluating early toxic effects of environmental chemicals and extrapolating from animal test systems across species and to man. The four formal sections are Aerotoxicology, Pharmacokinetics, Toxication-Detoxication, and Marine Pharmacology and Biomedicine; the work group is Molecular Pharmacology and Biochemistry.

Program

Aerotoxicology Section: This group has a mixture of research projects including studies on physiological and molecular mechanisms by which volatile chemicals affect the body, especially the lung, as well as studies of gross toxic effects of inhaled materials in the whole animal, and problems in generating various mixtures of particles, vapors, and gases for use in animal exposure chambers.

In the last year, research on toxicity and metabolism of solvents (especially benzene, xylene, and dichloromethane) and vinyl chloride has continued. We have studied the hepatic and pulmonary metabolisms of benzene and xylene in normal animals as well as animals pretreated with inducers and inhibitors of chemical-metabolizing systems in these tissues. Toxic responses of these control vs. pretreated animals to inhaled solvents have been compared with changes in rates of solvent metabolism by lung or liver from these two groups. Similar studies with dichloromethane have measured CO-hemoglobin levels in control vs. pretreated animals after DCM exposure and have compared these blood-CO changes with levels of chemical-metabolizing enzymes in liver and lung. The vinyl chloride experiments have involved animal exposures to high levels of vinyl chloride repeated daily for up to two weeks. The animal groups included controls and phenobarbital-pretreated animals. Enzymes metabolizing inhaled chemicals exist in the lung and other extrahepatic tissues as well as in liver. Success in manipulating these systems by stimulators or inhibitors is usually confined to the liver. Inhaled solvent effects on animals may be changed by changing metabolism of the solvents at sites of entry and in the body's major chemical-metabolizing organs (quantitatively, this is usually in the liver).

So far we have shifted whole body toxicity or toxic responses of animals to inhaled xylene, dichloromethane, and vinyl chloride by either inhibiting or stimulating the systems metabolizing these chemicals. These kinds of chemical-chemical, chemical-drug interactions will be explored further to help in identifying populations of people at greater than normal risk and synergistic effects which can perhaps be avoided.

Freon toxicities for the cardiovascular system have been studied further and more work in this area will occur when our new subsection in cardiovascular and respiratory physiology (with Drs. O'Neil and Van Stee) is fully activated. Cardiovascular and respiratory responses to inhaled chemicals and pollutants may be a part of the toxic actions of this group of environmental hazards as well as an early indicator of toxic responses to these materials. Animals with compromised respiratory and/or cardiovascular functions may be more sensitive to inhaled chemicals than normal animals. We have shown this to be true with freons and the cardiomyopathic hamster, and further studies of this nature will be made with mixtures of fluorocarbons, fluorocarbons plus other chemicals such as air pollutants, and air pollutants likely to increase as a result of the energy crisis and the greater utilization of non-petroleum energy sources.

Oven cleaners and constituents of spray can anti-perspirants were studied and it was shown that aerosolized oven cleaners produced no effects when inhaled by animals, whereas the inhalation of aluminum chlorohydrate produced lung lesions that were related to dose and/or time of exposure. The reversibility of these lesions and half-life of deposited aluminum in the lungs of animals inhaling aerosols of aluminum chlorohydrate have been studied: aluminum disappears from the lung, but the granulomas remain.

A contract to conduct chronic exposures of animals to selected pollutants has been negotiated with Becton-Dickinson and these studies are now beginning on-site. This collaborative on-site effort will maximize the effectiveness of our in-house inhalation toxicology studies in that development work, acute studies, and chronic studies can be closely coordinated. Animals exposed to selected chemicals or mixtures in the chronic-exposure facility can also be studied by groups in-house who are using sophisticated biochemical or toxicological test systems for toxic effects or studies on mechanisms of toxicity. The contract is primarily intended to identify chronic effects of mixtures of inhaled contaminants by classical inhalation toxicology technics (organ, body weight, histopathology); in-depth studies on differences between acute and chronic effects will be conducted largely in-house on exposed animals supplied by the contractor. Initial studies are concerned with sulfuric acid mist, ozone, and mixtures of the two. Synergistic effects will be studied with regard to dependence on concentration and ratios of the two pollutants.

A small contract to characterize the Red Tide aerosol has been negotiated with Florida State University. Inhaled aerosols of the Red Tide are respiratory irritants. The contract will characterize the particles in these aerosols and the possible role of particle vs. adsorbed toxin(s) in the irritant effects of Red Tide. Collaboration with the Marine Pharmacology Section is extensive as is interagency cooperation and information exchange at state and national levels. Both Dr. Drew and Dr. Bend have met with groups from many Florida State agencies as well as Federal agencies to discuss our program. Dr. Bend has attended two recent international meetings on Red Tide Research (at Tampa, Florida and MIT, Boston), and we have exchanged information with NHLI. See Marine Pharmacology Section for more details.

Marine Pharmacology and Biomedicine Section: This is a newly-formed section with several functions. Our in-house research efforts are devoted primarily to studies on uptake, distribution, metabolism, and excretion of pollutants by various marine species, and the role of metabolism of these pollutants in their storage and storage forms in these species. The pharmacokinetics of pollutant distribution in selected marine species is being determined and compared with mammals (see Pharmacokinetics Section) in the hope of improving mathematical models of and extrapolations of pollutant distributions from animals to man. Interactions of pollutants at these processes (uptake, distribution, storage, etc.) are also being studied to determine if mixtures of pollutants will lead to more storage of a pollutant than if the marine organism has to cope with only one chemical at a time. Effect of water temperature on these metabolic and storage processes will also be studied. A new laboratory (Whitney Marine Laboratory, St. Augustine, Florida) in Florida will be operated year round to complement the summer-only lab (Mount Desert Island Biological Laboratory, Maine) we have been using in this program over the last two to three years. So far we have shown that PCBs are stored in different tissues in different marine species and that the biological half-life of these pollutants correlates with the ability of the species to metabolize these chemicals. In lobsters, PCBs concentrate almost exclusively in the hepatopancreas, whereas in other species, significant PCB stores occur in fat as well as liver, and traces of PCBs can remain in fleshy tissue. Marine chemical metabolizing and detoxifying systems do not respond the same as mammalian systems to stimulators and inhibitors of these mammalian systems. In general, marine systems lack oxidative chemical metabolisms, though many conjugation reactions are present (e.g., glutathione conjugation, amino acid conjugations). Water soluble pollutants (2,4-D; 2,4,5-T) can thus be handled readily by most marine species, whereas lipophilic pollutants (DDT, PCBs) are often stored and accumulated. Stimulators of mammalian oxidative reactions (e.g., phenobarbital, DDT, PCB, TCDD) have little, if any, such effects in several marine species. What often occurs is that the low levels of these chemical-metabolizing systems present in some marine species (e.g., little skate) are inhibited by chemicals that are inducers of chemical oxidations in mammals. Thus,

the presence of DDT and PCB together will, we predict, lead to even greater accumulation by marine organisms of both pollutants than when either is present alone.

In addition to these studies, we will be initiating a small in-house research effort on the respiratory effects of the Red Tide (in collaboration with the Aerotoxicology Section). The nature of the aerosol and methods for generating this for experimental use in producing animals exposed to Red Tide aerosols will be the responsibility of the Aerotoxicology Section. The Marine Pharmacology Section will investigate the chemical and pharmacologic nature of the Red Tide toxin and whether this toxin plays any role in the respiratory irritant effects. Animals exposed to Red Tide toxins will be evaluated for effects using respiratory physiological measurements -- again in collaboration with the Aerotoxicology Section (with Dr. O'Neil). These plans were just recently discussed at the Red Tide Conference in Tampa, Florida, where it was determined that no such studies were contemplated by anyone identified as working in this area of marine science.

In addition to these research efforts, a survey of the marine biomedical research activity in the U. S. is being made. A conference was held in Washington, D. C., February 13-14 and scientists and directors of many of the marine science labs in the country attended. Recent research findings and summaries of activities at these labs were presented and there was an attempt to determine willingness and capability to be involved in a national program of marine biomedicine with emphasis on its human health impact. Dr. Bend of our Branch, Drs. Woods and Fowler of the Environmental Toxicology Branch, and Dr. Rall will be working together to try to develop a national program of marine research related to human health with input and collaboration from all other interested parties, such as other NIHs (NCI, NHLI, NICHD, NIAMDD, NINDS), NSF-Sea Grant program, NOAA, DOI, etc. The main concern of the NIEHS will be, however, to provide a focus for DHEW activities in marine biomedicine.

Pharmacokinetics Section: This section is conducting research in at least two major categories: (1) whole body distribution and modeling studies, and (2) uptake, storage, metabolism, and release of pollutants, etc., by isolated perfused organs. The kinetics of chemical disposition in whole body and isolated organs are being determined in order to construct mathematical models of these processes which can be used: (a) to better understand rate-limiting steps in these, (b) extrapolate distribution profiles of chemicals from one tissue to another, one species to another, and to man, and (c) predict tissue storage of pollutants from knowledge of exposures.

Efforts in the last year have concentrated on whole body distribution kinetics with selected PCBs, disposition of selected amines, as well as dieldrin and aldrin, by the isolated perfused lung, and disposition of selected PCBs by the isolated perfused liver. Pharmacokinetic modeling

has involved extensive collaboration with the Environmental Biometry Branch (especially Dr. Anderson) and groups at NIH, Bethesda, Maryland, (Drs. Dedrick and Zarharko, NCI, DCRT). The Environmental Biology and Chemistry Branch has been of tremendous help to this (and many other Branch programs) in identification of pollutant metabolites and in supplying certain reference compounds (labeled and unlabeled) for use. Role of PCB metabolism in determining whole body and tissue half-life, nature and number of storage compartments, and fate of different PCB isomers has been elucidated and further studies on the structure-activity relationships of PCB fate using selected PCB isomers continues. Most PCBs end up in fatty tissues, but clearance from the body depends on metabolism. Appreciable storage of PCBs in skin was found and may explain the dermal toxicity of ingested PCBs that is seen in many species.

Studies suggest that rapidly metabolized PCBs will not be stored or accumulated in animals or man and that use of such PCBs could markedly reduce the environmental health hazards of this class of chemicals. Lung storage of most PCB's is very slight, but the chlorinated pesticides dieldrin and aldrin do accumulate in lung. Pharmacokinetic studies of this process showed that once exposure is terminated, these chlorinated hydrocarbons efflux rapidly from the lung and therefore pose much less hazard than with most chemical epoxides where tissue storage may be very long term due to reaction of the epoxide with tissue macromolecules.

Amine storage in the lung is often extensive, but several processes of uptake and efflux were identified. Some of these involve passive diffusion and therefore are blood-concentration dependent. Some amines appear to be accumulated by lungs by more specific mechanisms and these have been studied in more detail. Active or facilitated transport mechanisms offer the chance for competition between amines for uptake and release and offer possibilities for chemical or drug interactions and synergistic effects. Several vasoactive, endogenous hormones are amines whose uptake or release by the lung is vital to maintaining homeostasis and proper lung and cardiovascular functions. Competition between chemicals (exogenous) and hormones (endogenous) for lung uptake or efflux sites could therefore be an important mechanism for lung toxicity and cardiovascular effects of pollutants. Several valuable therapeutic agents (tranquilizers, analgesics, hypnotics) are stored in lung in appreciable quantities. Competition for storage or release of these drugs by amine pollutants could have serious effects on drug toxicity and action in patients. So far we have shown that drugs and vasoactive hormones can compete for lung uptake and efflux processes, and this is being extended to pollutant-drug-hormone interactions.

Another group of compounds regulated by the lung are the hormones, prostaglandins. These lipids are synthesized, stored, and metabolized in lung, and the lung plays a very important role in regulating circulating levels of these very active materials. Effects of pollutants on prostaglandin disposition by the lung are being investigated. We have exposed animals

to various oxidant gases and then studied prostaglandin synthesis and metabolism by lungs from these exposed animals. Effects of oxygen, ozone, SO_x and NO_x have been studied and the results suggest that prostaglandin disposition by lung can be affected by breathing at least some of these pollutants. The time-concentration relationships of these effects are being studied in greater detail so as to better define the practical limits of these changes and their possible role in lung disease initiated by these pollutants.

The Pharmacokinetics Section will be working with the Marine Pharmacology Section in modeling for pollutant distributions in marine vs. mammalian species. This collaboration has been referred to above.

Toxication-Detoxication Section: This is a research program concerned with the role of chemical metabolism in the production of tissue damage or toxicity. Metabolisms leading to increased toxicity may be balanced by reactions with detoxifying systems. This balance of toxication-detoxication mechanisms may be upset by pollutants, pathology, age, species, or be different in one tissue from another. Chemical-metabolizing systems have been or are being characterized in tissue most likely to contact the environment -- skin, intestine, lung, and liver. Characterization includes substrate and inhibitor specificity, ability to be stimulated or induced, and measurements of components of multicomponent systems (e.g., P-450 concentrations and types). Fractionation and purification of epoxide-producing and -detoxifying systems is being carried out with lung, liver, intestine, and skin. Much emphasis is being placed on the relative activities of MFOs (P-450 dependent), epoxide hydrase, glutathione-S-epoxide transferases, and N-oxidases in various tissues and at different times in the perinatal period to identify when and in what tissue an imbalance in epoxide or active metabolite production and destruction may occur. We have shown that the balance of epoxide production and destruction is abnormal (compared to adult tissue levels) at several times before and just after birth in several tissues. We have shown that effectors of adult enzyme systems behave differently in the fetus and newborn (e.g., effects of Hg²⁺ on N-oxidation) and that pregnancy can affect systems in liver and lung quite differently (N-oxidation and MFOs in lung, but not in liver, increase). Some of these results were presented in Tübingen, Germany and Milan, Italy in June 1974 (International Symposium on Perinatal Pharmacology) and at the Society of Toxicology Meetings in March 1975.

Studies of chemical-metabolizing systems in different tissues in different species include comparisons of different mammalian species (mice, rats, hamsters, rabbits most commonly), as well as with marine species (in collaboration with Marine Pharmacology Section), and with birds (in collaboration with investigators at Mount Desert Island Biological Laboratory as well as with Dr. Pan of Fish and Wildlife Service, DOI). Most marine species and birds have much lower ability to oxidize chemicals, though non-oxidative (e.g., conjugating) systems are often present at appreciable levels in several tissues. Thus, both marine species and birds will be

likely to dispose quickly of water soluble pollutants (like 2,4-D; 2,4,5-T), but accumulate lipid soluble pollutants (DDT, PCBs, TCDD). Such may be useful data in designing environmentally safer pesticides or selective poisons (e.g., for bird control, of interest to DOI).

Most extrahepatic chemical-metabolizing systems resemble those of the same type in liver. Certain peculiarities are being explored, however. Thus, the lung MFOs seem especially able to metabolize organic solvents and especially poor in most drug-metabolizing abilities. Purification of the lung MFO systems suggests these differences are not explainable in terms of cytochromes, but rather the associated flavoprotein and lipid fractions. In skin, the MFO system seems especially unlike liver in its response to polycyclic hydrocarbon inducers as well as its concurrent content of non-P-450 hemoproteins. These studies may help explain the sensitivity of skin and lung to certain types of chemicals whose toxic effects depend on metabolic activation (e.g., solvents, polycyclic hydrocarbons).

Metabolic activation of chemicals as a key process in toxicity was the subject of a two-day workshop in collaboration with the Environmental Mutagenesis Branch. Several scientists who are leaders in the field of chemical metabolism or mutagenesis met together to discuss how best to obtain and measure metabolic activation of chemicals to mutagens. This workshop will be followed up by a conference this fall or winter wherein about a score of leaders in metabolism or genetics will seek to describe not only the state of the art, but what is needed in both fields to get a better feel for the role of chemical metabolism in producing genetic damage.

Molecular Pharmacology and Biochemistry: This work group is involved in several studies relating to many other sections of the Branch. Work on the secretion of lung surfactant has concerned the role of the concentric lamellar organelle (CLO) as a precursor of lung surfactant and the role of enzymes of the CLO in the release of surfactant from these CLOs. Enzymes of the surfactant (lung) washout fraction have also been studied, as have enzymes of the lung macrophage in a program designed to better understand chemical alterations that can occur outside the lung parenchyma (i.e., to inhaled chemicals before they are absorbed into the body). These studies tie in with the Pharmacokinetics Section (IPL especially) and the Toxication-Detoxication Section.

Also being studied are chemical-macromolecule interactions. Membrane associated enzyme systems can incorporate drugs and chemicals into peptides as well as coenzymes. Thus, NAD glycohydrolase has been studied in its interaction with hydrazines to form DPN-analogs. In the lung this reaction may be responsible for both therapeutic (INH as tuberculostatic) and toxic effects (hydrazide-induced lung toxicity). This enzyme (NAD glycohydrolase) may serve as a marker of cell membrane function as well, and its behavior in normal vs. tumor cells is being studied. Another

membrane-bound enzyme system, transglutaminase, is being studied for its role in membrane function and alterations. Polyamines can be incorporated into membranes via transglutaminase and this may serve as a prototype for chemical-induced membrane changes, chemical-induced allergenic reactions, etc. Again this enzyme may serve as a marker for cell membrane changes after chemical injury or during neoplasia. Membrane glycoproteins are being studied as cell receptors -- both for chemical actions and hormone-induced changes in membranes. Some membrane glycoproteins are enzymes and changes in membrane protein can be correlated with enzyme function. Membrane glycoproteins are also involved in things like contact inhibition (important in neoplasia), and alterations in this process may precede cell damage and transformation.

Another program in this group seeks to study the interaction of epoxides with nucleic acids and what role the epoxide-DNA or epoxide-RNA complex may play in regulation of protein synthesis, DNA repair, etc. Most studies to date have concentrated on the formation of epoxide-DNA adducts, not on the biological activity of these adducts. This program is working closely with the Toxication-Detoxication Section (especially the group concerned with epoxide metabolism) and is collaborating with groups at NIH, Bethesda (Dr. Jerina, LC, NIAMDD, who will also be working with the Marine Pharmacology Section and the Toxication-Detoxication Section on epoxide metabolisms in various tissues of marine and mammalian species).

Facilities

Dr. Eling and his group (Pharmacokinetics Section) have been relocated in building 14. This allows better coordination of efforts in this section as well as productive collaboration with Dr. Drew's section (aerotoxicology, respiratory physiology, cardiovascular physiology) and members of the Toxication-Detoxication Section (Dr. Bend, comparing xenobiotic metabolism by isolated perfused lung and cell fractions of lungs) and the Molecular Pharmacology and Biochemistry Group (Dr. Hook, studying benzpyrene binding by lung surfactant, enzyme composition of lung surfactant, surfactant release). The contract for long-term inhalation toxicology studies has been negotiated and these studies are being conducted in the west half of building 14. This close proximity of contractor to Dr. Drew's group allows close collaboration of acute and chronic studies and design of new aerotoxicology methodology.

Personnel

Dr. Drew's group has added two scientists -- Dr. John O'Neil (respiratory physiology) and Dr. Van Stee (cardiovascular and inhalation toxicology). Drs. P. Voytek and K. Ku (Visiting Fellow with Dr. Voytek) transferred to the Branch from the Environmental Toxicology Branch (epoxide-DNA adducts). Visiting Fellows added this year were Dr. Z. Ben Zvi (with Dr. Bend), Dr. E. Arinc (with Dr. Philpot), and Dr. J. M. Patel (with Dr. Harper). New technicians were Ms. H. Hawkins (with Dr. Eling, replaced

Mr. Postlethwait), Ms. E. Smart (with Dr. Chhabra), and Mr. W. Gibson (with Dr. Drew, replaced Mr. Lores). Special program members included new replacements in the Stay-in-School program -- Ms. V. Hyatt, Ms. B. Merritt, Mr. D. Jones, Mr. C. Raines, Mr. R. Ranson, Ms. J. Younger, and temporary technicians -- Ms. S. Vicinus and Mr. R. Walden. A new Visiting Associate, Dr. R. Mirel, is working with Dr. DiAugustine.

Losses from the Branch included Dr. G. Hasler (Staff Associate), Dr. S. Hwang (Staff Fellow), Dr. R. Pickett (Visiting Fellow), Mr. E. Postlethwait (technician with Dr. Eling), Mr. E. Lores (technician with Dr. Drew); several stay-in-school members -- Mr. B. Armstrong, Mr. C. Daniel, Mr. E. Jackson, Mr. W. Lee, Mr. K. Moore, Mr. D. Prince, and Mr. E. Slade; and temporary technicians -- Ms. S. Vicinus and Mr. R. Walden.

Other Activities

Several of our professional staff serve on committees and give lectures at local universities or help with committees in other Federal agencies and professional societies. The listing below is meant to be complete, but may suffer omissions:

Dr. J. R. Bend: Presented paper at the 12th Annual Meeting of the Interagency Collaborative Group on Environmental Carcinogenesis, Washington, D.C. (September 1974); invited speaker at the Florida Red Tide Conference, Sarasota, Florida (October 1974) and at the 14th Annual Meeting of the Society of Toxicology, Williamsburg, Virginia (March 1975); invited lecturer at the Department of Physiology, School of Medicine, University of Pennsylvania, Philadelphia, Pa. (January 1975); prepared book chapter for the Handbook of Physiology, American Physiological Society; NIEHS representative on two subgroups of the CEQ/OMB Interagency Working Group on Health and Environmental Effects of Energy Use (Subgroup A - Physical Environment [non-pollutant and non-biological effects], Subgroup B - Ecological Effects); helped organize the NIEHS-sponsored Marine Biomedical Research Meeting, Smithsonian Institution, Washington, D. C. (February 1975); member of workshop on "In vitro metabolic activity of chemicals to mutagens," NIEHS (January 1975).

Dr. R. S. Chhabra: member of the Library Committee, NIEHS.

Dr. R. P. DiAugustine: Invited lecturer at Department of Pathology, Duke University Medical Center and the Environmental Pulmonary Conference, Duke University Medical Center; member of NIEHS Medical Science Lectures Committee.

Dr. R. T. Drew: Invited speaker for Cosmetic Toiletries and Fragrance Association Inter-Industry Aerosol Safety Conference (September 1974); testified before Consumer Products Safety Commission (February 1974, March 1974) and FDA-OTC Panel on Antiperspirants (December 1974); member of NIEHS Radiation Safety Committee, NIEHS Energy Committee, NCI Program

Review Committee, ACGIH Air Sampling Instruments Committee, and Society of Plastics Toxicology Committee; contract officer for Becton-Dickinson and Red Tide.

Dr. J. R. Fouts: Adjunct professor at University of North Carolina and North Carolina State University, visiting lecturer at Duke University -- various committees and lecturer to medical and graduate students at all three universities; member of Dental Research Center's Scientific Review Committee (University of North Carolina, Chapel Hill); editorial board member of Xenobiotica, Cancer Research, and Journal of Toxicology and Environmental Health; editor with Dr. Hart of Chemico-Biological Interactions (Elsevier Press); committee memberships include Nominating Committee (Society of Toxicology), Anticonvulsant Drugs and Epilepsy Advisory Committees (NINDS, NIH), Basic Pharmacology Advisory Committee (Pharmaceutical Manufacturers' Association Foundation), Drug-Interactions Contract Review (NIDA, ADAMHA), and Office of Pesticide Programs, contract review (EPA); newly elected chairman, Division of Drug Metabolism, American Society for Pharmacology and Experimental Therapeutics; lectures were given at University of Tübingen, West Germany (June 1974), University of North Carolina, School of Pharmacy (September 1974), Bowman Gray Medical School (October 1974), Clinical Ecology Seminar, Denver (December 1974), and FASEB Symposium, Atlantic City (April 1975).

Dr. C. Harper: Adjunct assistant professor at University of North Carolina, Department of Pharmacology; lectures to medical and graduate students in departmental courses in pharmacology.

Dr. L. G. Hart: Adjunct associate professor, Department of Pharmacology, University of North Carolina; lectures presented to medical and graduate students of University of North Carolina; seminar presented at Department of Pharmacology, University of North Carolina; member of NIEHS Safety Committee (involved in formation of this committee); NIEHS project director for coordinating efforts in three task areas under the "health effects of the non-nuclear energy technologies" program; associate managing editor (U.S.) for Chemico-Biological Interactions, editorial board member of Environmental Health Perspectives, reviewer for Biochemical Pharmacology.

Dr. G. R. Hasler: Member of NIEHS Safety Committee.

Dr. G. E. R. Hook: Presented seminar at Department of Biochemistry and Medicine, Duke University (March 1975); co-managing editor of Environmental Health Perspectives, editorial board member of Environmental Health Perspectives.

Dr. M. O. James: Invited lecturer at Biochemistry Department of St. Mary's Hospital Medical School, London, England (December 1974).

Dr. R. M. Philpot: Adjunct assistant professor, North Carolina State University; member of NIEHS editorial board, ad hoc committee for "inbred mouse strains"; member of the workshop on "In vitro metabolic activation of chemicals to mutagens," NIEHS (January 1975).

Ms. R. J. Pohl: NIEHS Safety Committee Building Monitor for Building 14.

Dr. P. E. Voytek: Lectured at Department of Medicine, Duke University (February 1975); presented seminar at Department of Biochemistry, University of Vermont (July 1974), Department of Biochemistry, University of North Carolina (February 1975); editorial board member of NIEHS in-house review.

Project No.: Z01 ES 80001-04 PB

1. Pharmacology Branch

2.

3. Research Triangle Park, N. C.

PHS-NIH

Individual Project Report

July 1, 1974 through June 30, 1975

PROJECT TITLE: "Microsomal Mixed-Function Oxidase Systems: Specificity and Function"

PREVIOUS SERIAL NUMBER: NIEHS-P-008

PRINCIPAL INVESTIGATOR: R. M. Philpot, Ph.D.

OTHER INVESTIGATORS: Emel Arinc, Ph.D. and J. R. Bend, Ph.D.

COOPERATING UNITS: None

MAN YEARS

Total: 4.5

Professional: 2.0

Other: 2.5

PROJECT DESCRIPTION

OBJECTIVES: The microsomal mixed-function oxidase (MFO) system of mammals exhibits species, sex, and tissue variations. Treatment of animals with various pharmaceutical agents, environmental pollutants, and other exogenous compounds can bring about changes in the MFO system(s). Both stimulation and inhibition of these systems can be elicited, and in some cases this can be accomplished with the same compound. It is not understood if these changes are due to direct effects on the major components of the MFO, to alterations in the membrane environment, or to changes in the concentrations or kinds of endogenous compounds that interact with the MFO.

The objective of this project is to increase our understanding of the factors responsible for the variances noted among mixed-function oxidase systems from different sources. This objective is being pursued via several approaches:

1. Tissue Differences: The MFO systems from rabbit liver and lung are being solubilized and separated into their component parts. These components (cytochrome P-450, TPNH cytochrome c reductase and lipid) and the enzymatic activities obtained by component recombination are being

compared.

2. Cytochrome P-450 Differences: Rabbit pulmonary and hepatic cytochrome P-450's are being purified and compared.

3. Differences in Response to In Vivo Treatment: The effects of in vivo administration of 3-methylcholanthrene (3-MC) on the MFO in adult rabbit and rat liver are being studied.

METHODS EMPLOYED: The components of the liver MFO systems are solubilized and separated by the methods developed by Lu and co-workers. These methods have been modified for application to the rabbit pulmonary MFO system. Pulmonary cytochrome P-450 is being purified by the combination of existing techniques for liver cytochrome purification and the development of new techniques. Spectral analysis of cytochrome fractions is carried out according to methods developed in this laboratory.

MAJOR FINDINGS: 1. The pulmonary MFO system has been solubilized and separated into three components: cytochrome P-450, TPNH cytochrome c reductase and lipid. Hydroxylase activity is obtained when these components are recombined. TPNH cytochrome c reductase prepared from pulmonary microsomes by the method used to obtain solubilized reductase from the liver (deoxycholate digestion) is not active when combined with pulmonary cytochrome and lipid in reconstituting MFO activity. However, reductase which is active in reconstituted systems can be prepared from the lung by cholate digestion. The N-demethylation of benzphetamine and the O-deethylation of 7-ethoxycoumarin proceed at a higher rate in the reconstituted pulmonary system than they do in the reconstituted hepatic system. This is also true in microsomal systems. Thus, the pulmonary MFO system appears to be more effective than the hepatic MFO system in dealing with some substrates. It has also been shown in the pulmonary reconstituted MFO system that the concentration of TPNH cytochrome c reductase required for maximum activity is dependent on the substrate employed.

2. Pulmonary cytochrome P-450 has been purified 25-fold from microsomes. Preparations with specific activities of 5 to 5.5 nmoles cytochrome per mg protein have been obtained by detergent digestion, fractionation with ammonium sulfate and chromatography on DEAE cellulose and hydroxyapatite. These preparations are essentially free of cytochromes b₅ and P-420 and TPNH cytochrome c reductase. Similar preparations of hepatic cytochrome P-450 from untreated rabbits are being obtained.

3. Treatment of rabbits with 3-MC results in the induction of cytochrome P-448 but not benzpyrene hydroxylase activity. In the rat both the cytochrome and benzpyrene hydroxylase are induced. Experiments using reconstituted systems show that the cytochrome fraction is responsible for the lack of benzpyrene hydroxylase activity in the 3-MC-induced rabbit.

It has been observed that rabbit cytochrome P-448 contains more "high-spin" heme than does rat cytochrome P-448. This suggests that the cytochrome from the rabbit may be inactive due to the presence of a bound endogenous substrate. The "high-spin" state of the rabbit cytochrome can be reversed by the addition of caffeine but this does not result in increases in benzpyrene hydroxylase activity. Caffeine does not act as an inhibitor when added to systems containing rat cytochrome P-448.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE:

A full understanding of mixed-function oxidase systems is required if drug interaction, drug metabolism, disposition of ingested or inhaled environmental pollutants, and pesticide detoxification are to be fully comprehended.

This problem is complicated by the multitude of functional differences and responses exhibited by MFO systems from various sources. Identification of the factors which control the activities of MFO systems is essential to our understanding and manipulation of this complex biological process.

The methods by which the lung deals with inhaled substances is of particular importance. The lung is a target organ for the action of polycyclic hydrocarbons which are an important class of environmental pollutants. It has been hypothesized that these compounds must be metabolized by the MFO system in order to become effective as carcinogens and/or mutagens. The differences between the lung and liver systems in dealing with these compounds may provide part of the explanation for target organ specificity.

PROPOSED COURSE: The present work involving the solubilization, reconstitution and determination of substrate specificities of the MFO systems from rabbit liver and lung will be continued. The effect of 3-methylcholanthrene in rat and rabbit will be further examined in an effort to identify the factors responsible for the differences noted in responses.

KEYWORD DESCRIPTORS

Liver and lung microsomes, mixed-function oxidases, solubilization of MFO, P-450, P-448, TPNH cytochrome c reductase, lipid, 3-MC, difference spectra, rat vs. rabbit MFO

PUBLICATIONS

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Philpot, R. M., Arinc, E., and Fouts, J. R.: Reconstitution of the rabbit pulmonary microsomal mixed-function oxidase system from solubilized components. Drug Metab. Disp. in press, 1975.

Philpot, R. M. and Bend, J. R.: Benzpyrene hydroxylase activity in hepatic microsomal and solubilized systems containing rabbit or rat cytochrome P-448 or P-450. Life Sciences 16: 985-998, 1975.

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Project No.: Z01 ES 80002-05 PB

1. Pharmacology Branch
- 2.
3. Research Triangle Park, N. C.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

PROJECT TITLE: "Enzymes Metabolizing Chemicals: Chemical and Physiological Effectors of These Systems"

PREVIOUS SERIAL NUMBER: NIEHS-P-001

PRINCIPAL INVESTIGATORS: T. R. Devereux, M. S. and J. R. Fouts, Ph.D.

OTHER INVESTIGATOR: R. M. Philpot, Ph.D.

COOPERATING UNITS: Environmental Biometry Branch, Environmental Biology and Chemistry Branch

MAN YEARS

Total:	1.0
Professional:	0.8
Other:	0.2

PROJECT DESCRIPTION

OBJECTIVES: This effort is directed towards understanding how various chemicals (pollutants, steroids, etc.) and physiological changes (age, pregnancy, nutrition, disease) affect the way xenobiotics are metabolized by the body. Using N-demethylation, N-hydroxylation and N-oxidation as model systems, detoxication and possible toxication-metabolic pathways of the same substance are compared. Present efforts are devoted to studies on activities of these enzymic pathways in liver and lung microsomes of pregnant rabbits as compared to non-pregnant rabbits and to rabbits which have been treated with certain steroids. Mechanisms of metal ion effects on the pulmonary N-oxidase system are also under investigation. A method for studying N-hydroxylation of primary and secondary amines is being set up at the present time.

METHODS EMPLOYED: Most work has been done on microsomal preparations isolated from liver and lung of rabbits. These microsomes have been used to study the metabolism of different xenobiotics or the amount or activity of key steps in the MFO-P-450-dependent system as well as the P-450-independent N-oxidation system. The N-oxidase enzyme has

been partially purified from lung and liver by classical techniques of enzyme purification -- solubilization with detergents, concentration by selective precipitation with $(\text{NH}_4)_2\text{SO}_4$, etc. Spectrophotometric methods are used to study cytochrome P-450 and to quantify metabolites of substrates added to microsomes or purified enzyme systems.

MAJOR FINDINGS: Increased levels of N-demethylase and N-oxidase activities were seen in lung but not liver microsomes of rabbits during the latter third of pregnancy. Non-pregnant rabbits were treated with certain steroids which have high plasma levels during late pregnancy (testosterone, corticosteroids). Pretreatment of adult non-pregnant rabbits with dexamethasone or hydrocortisone (corticosteroids) caused increases of 50% or greater in N-demethylation, N-oxidation and cytochrome P-450 concentration in both liver and lung microsomes. Pretreatment of rabbits with deoxycorticosterone (mineralocorticoid) caused increases of about 50% in N-demethylation, N-oxidation and cytochrome P-450 in lung microsomes, but there was no change in these activities in liver microsomes. Treatment of rabbits with testosterone had no effect on these activities in liver or lung microsomes. The solubilized and partially purified rabbit lung N-oxidase was studied to learn why Hg^{+2} stimulated the microsomal lung N-oxidase but inhibited the N-oxidase in liver microsomes. A heat-stable, non-dialyzable factor possibly flavin-bound, seems to be involved. This "activation factor" is present in liver as well as lung but only effects mercury stimulation of the N-oxidase in lung.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: These research projects seek to understand some of the factors that affect xenobiotic metabolism in tissues in contact with the environment. Comparisons of lung and liver systems have been made to see where differences exist and what these might mean in toxication-detoxication mechanisms of the body. Studies of changes caused by pregnancy or steroids in toxication versus detoxication pathways may aid our identifying and understanding stages in life when the body is more susceptible to toxic agents. These studies, as well as those into the mechanisms of action of metal ions on N-oxidation in lung and liver and the comparisons of similar enzyme systems (e.g., N-hydroxylation vs. N-oxidation) in different tissues, may lead us to a better understanding of the balance between toxication and detoxication mechanisms and the varied ways chemicals and physiological changes can alter these systems and this balance.

PROPOSED COURSE: Further studies on various chemical (selective stimulators and inhibitors of N-oxidation) and physiological stresses (disease, e.g., diabetes) to see how xenobiotic metabolism is affected especially in the lung. Study of N-hydroxylation of primary and secondary amines to compare mechanisms of this oxidation with N-oxidation of tertiary amines by lung and to understand the toxicological importance of N-hydroxylation and N-oxidation.

KEYWORD DESCRIPTORS

Lung; xenobiotic metabolism; toxication-detoxication mechanisms; N-hydroxylation; N-oxidation; cytochrome P-450; pregnancy; steroids; metals.

PUBLICATIONS

Devereux, T. R. and Fouts, J. R.: Effect of pregnancy or treatment with certain steroids on N,N-demethylaniline demethylation and N-oxidation by rabbit liver or lung microsomes. Drug Metab. Disp. in press.

Devereux, T. R. and Fouts, J. R.: Effects of pregnancy and certain steroids on demethylation and N-oxidation by rabbit lung microsomes. Toxicol. Appl. Pharmacol. Presented at Society of Toxicology Meetings, March, 1975. (Abstract)

Project No.: Z01 ES 80003-02 PB

1. Pharmacology Branch
- 2.
3. Research Triangle Park, N. C.

PHS-NIH

Individual Project Report

July 1, 1974 through June 30, 1975

PROJECT TITLE: "Microsomal Drug and Chemical Metabolizing Enzyme Activity in Skin and Its Response to Environmental Agents"

PREVIOUS SERIAL NUMBER: NIEHS-P-005

PRINCIPAL INVESTIGATORS: R. J. Poh1, B. A., J. R. Fouts, Ph.D., and R. M. Philpot, Ph.D.

OTHER INVESTIGATORS: None

COOPERATING UNIT: Environmental Biometry Branch

MAN YEARS

Total:	1.0
Professional:	0.8
Other:	0.2

PROJECT DESCRIPTION

OBJECTIVES: To isolate microsomal fractions from skin, characterize the xenobiotic-metabolizing enzymes associated with those fractions and elucidate the role of these enzymes in toxic responses of skin to environmental agents.

METHODS EMPLOYED: Mouse skin was disrupted in 0.5 gm aliquots, by four 5-second bursts with a Polytron, and homogenized in a conical homogenizer. Microsomal fractions were isolated by standard procedures. Cytochrome P-450 content was estimated from reduced CO difference spectra. Contamination by other hemochromes was identified by a variety of spectral difference techniques. 7-Ethoxycoumarin de-ethylating activity was measured by recording the appearance of the fluorescent metabolite, umbelliferone. Benzpyrene and aniline hydroxylase activities were assayed by standard techniques. Reduction of cytochrome c by NADPH was followed spectro-photometrically.

MAJOR FINDINGS: The hemochrome content of the skin microsomal preparations was identified and roughly quantified in nmoles/mg protein: cytochrome b₅, 0.03; cytochrome P-450, 0.005; cytochrome P-42C, 0.015; cytochrome oxidase, 0.02; hemoglobin, trace; methemoglobin, not detectable. Aniline hydroxylase

activity in the skin microsomal preparations was 0.25 nmoles/min/mg protein and NADPH-cytochrome c reductase was 7.9 nmoles/min/mg protein. Induction of cytochrome P-450 content, 7-ethoxycoumarin de-ethylase and benzyrene hydroxylase activities by topical application of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) was demonstrated. After cholate solubilization of the microsomes and ammonium sulfate fractionation, a wavelength peak shift to 447 nm was seen in the cytochrome P-450 spectra from TCDD-treated mice. Topical application of 3-methylcholanthrene had no effect under the treatment regime used.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Skin comes in contact with a large number of foreign chemicals found in cosmetics, cleaning compounds, polluted air and water, synthetic and "easy-care" fabrics and some occupational environments. Both localized and systemic toxicity of these chemicals may be mediated by the balance of toxicating and detoxicating mechanisms in the skin, including xenobiotic-metabolizing enzymes. The study of these enzymes and their responses to both chemical and physical factors will lead to a better understanding of the total body protective systems toward foreign chemicals.

PROPOSED COURSE: A survey of skin mixed-function oxidase systems in several species and strains of animals will be made to determine the animal of choice for continued investigation. Continued efforts will be made to improve the yield and purity of microsomal preparations. A systematic study will be made of the effects of MFO inhibitors and inducers (many of which are environmental contaminants), age, sex, and physiological stresses on skin enzyme activities and their response to environmental chemicals and radiation of various types.

KEYWORD DESCRIPTORS

Skin; mixed-function oxidase; cytochrome P-450; xenobiotic metabolism; difference spectroscopy; microsomes; enzyme induction; dermal toxicity.

PUBLICATIONS

Pohl, R. J., Philpot, R. M., and Fouts, J. R.: Cytochrome P-450 content and mixed-function oxidase activity in microsomes isolated from mouse skin. The Pharmacologist 16: 322, 1974. (Abstract)

Project No.: Z01 ES 80004-03 PB

1. Pharmacology Branch
- 2.
3. Research Triangle Park, N. C.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

PROJECT TITLE: "Study of Factors Affecting Toxication-Detoxication of Xenobiotics in Various Tissues, Especially Intestine"

PREVIOUS SERIAL NUMBER: NIEHS-P-004

PRINCIPAL INVESTIGATORS: R. S. Chhabra, Ph.D. and J. M. Tredger, Ph.D.

OTHER INVESTIGATORS: J. R. Fouts, Ph.D., R. M. Philpot, Ph.D., and Elizabeth Smart, B. S.

COOPERATING UNITS: Environmental Biometry Branch, Environmental Biology and Chemistry Branch

MAN YEARS

Total:	2.8
Professional:	2.0
Other:	0.8

PROJECT DESCRIPTION

OBJECTIVES: To study the effects of various physiological (age, sex, nutrition, hormones), genetic (animal species, strain), and environmental (exposure to insecticides, carcinogenic polycyclic hydrocarbons and heavy metals) factors on the enzymes metabolizing xenobiotics in various tissues.

There are three sub-projects currently under study: (1) Hormonal regulations of xenobiotic metabolizing enzymes in various species and strains of laboratory animals; (2) Comprehensive study of xenobiotic metabolizing enzyme systems in rabbit small intestine and their comparison with similar systems in liver, effect of environmental pollutants on those systems; and (3) genetic regulation of induction of xenobiotic metabolizing enzymes in various strains of mice.

METHODS EMPLOYED: Microsomal fractions were isolated by differential centrifugation. Spectrophotometric and spectrophotofluorometric techniques were used for the assays of xenobiotic metabolizing enzymes.

MAJOR FINDINGS: 1. (a) Studies on hormonal regulation of ethylmorphine N-demethylase, aniline hydroxylase, and benzpyrene hydroxylase in mice indicated that adrenalectomy, castration or adrenalectomy plus castration does not lower the basal (uninduced) activities of these enzymes. Also the magnitude of stimulation of hepatic xenobiotic metabolizing enzymes by pretreatment of mice with DDT, BP (benzpyrene), 3-MC (3-methylcholanthrene) or phenobarbital in mice without adrenals, testes or both was not less than in sham-operated control mice (as does occur in rats) but instead was somewhat higher. These findings suggest that there is a species variation in hormonal regulation of hepatic xenobiotic metabolism in rats and mice.

(b) Hepatic microsomes from male rats metabolize xenobiotics faster than hepatic microsomes from female rats. Sex differences in the metabolism of xenobiotics may be specific to only some organs or tissues since enzyme preparations from rat lung and intestines did not show sex differences in the metabolism of various xenobiotic substrates. Rat kidney microsomes exhibited sex differences in rates of hydroxylation of BP and glucuronidation of p-nitrophenol, though in contrast to liver, the rates of BP and PNP metabolism were higher in kidneys from female than from male rats. The sex differences in both hepatic and renal microsomal UDP-glucuronyl transferase activity existed even after maximal activation of the enzyme by Triton X-100.

2. Various laboratory animal species were used to select a model animal for more detailed study of xenobiotic metabolizing enzymes in small intestine. In intestines from mice, rats, guinea pigs, and hamster, either some of the enzymes were absent or had very low activity which would require very sensitive methods for detection. Rabbit emerged as the best animal model to study xenobiotic metabolisms in small intestine because all xenobiotic metabolizing enzymes studied were present in easily measurable quantities. A distribution study of some of the xenobiotic metabolizing enzymes along the entire length of small intestine showed that maximum activity of these enzymes is located in the proximal 24 inches of the small intestine. Rabbit intestinal xenobiotic metabolizing enzymes required reduced nicotinamide adenine dinucleotide phosphate and oxygen for maximum activity. In general the protein vs. activity, time vs. activity, substrate concentration vs. activity, and pH vs. activity curves of aniline hydroxylase, BP hydroxylase, and aminopyrine N-demethylase were similar in microsomes from small intestine and liver. Cytochrome c, SKF 525-A, and CO inhibited microsomal mixed-function oxidases in small intestine as well as in liver.

Studies of the post-natal development of xenobiotic metabolizing enzymes and cytochrome P-450 in the small intestines and livers of rabbits showed that, in general, the patterns of development of drug metabolizing enzymes in the small intestine were similar to those seen in the liver. Some of the enzymes studied were detectable during the first week after birth and by the end of the second week all enzymes could be assayed. During the third week after birth, small increases in the

activities of the enzymes were observed, but amounts remained less than 50% of those found in the adult. However, 4 weeks after birth, 2 to 5-fold increases in activity occurred and at this time equaled or exceeded those activities in the adult small intestine. For the next 7 weeks the activities of the intestinal enzymes fluctuated (usually falling below adult values at about 40 to 50 days post-partum), but then rising to adult levels by 7 to 11 weeks of age. The maturation of xenobiotic metabolizing enzyme activity was accompanied by a proportional change in the content of cytochrome P-450 in intestinal microsomes. Hemoglobin from adult or newborn rabbits did not catalyze aniline metabolism. Thus, the relatively high aniline hydroxylase of newborn liver was not due to contamination by hemoglobin.

3. Various microsomal enzyme inducers such as DDT, benzpyrene, 3-MC, tetrachlorodibenzo dioxin (TCDD), or phenobarbital were studied for their effects on hepatic microsomal mixed-function oxidases and cytochrome P-450 content in mice genetically responsive (C57BL/6J) and resistant (DBA/2J) to induction by BP of aryl hydrocarbon hydroxylase (AHH, benzpyrene hydroxylase). Benzpyrene or 3-MC administration to mice stimulated hepatic AHH activity 2- to 3-fold in C57BL/6J but not in DBA/2J mice. TCDD stimulated AHH activity in liver of both mouse strains 6- to 8- fold. This increase was accompanied by a delayed shift in the peak of cytochrome P-450-CO-difference spectrum from 450 to 448 nm. This study suggests that genetic resistance of AHH to stimulation in DBA/2J mice is influenced by the type of inducer used.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE:

Species and strain differences in metabolism of and response to pollutants by xenobiotic metabolizing systems may explain both species and strain specific toxicities of pollutants and interactions of pollutants with drugs and chemicals. Pollutants in food and water as well as in air contact intestine and intestinal enzymes may be a first line of defense of the body against such materials and subsequently those enzymes may influence the bioavailability of toxic agents.

PROPOSED COURSE: Effect of carcinogenic polycyclic hydrocarbons on xenobiotic metabolism in genetically resistant and susceptible strains of mice will be taken a step further by studying the inducibility of these enzymes located in the nuclear (vs. microsomal) membranes. Effect of hormones on inducibility of AHH by 3-MC in resistant mice will also be studied.

Plans are to study the effect of various enzyme inducers (such as DDT, BP, 3-MC, TCDD, PCB's) and inhibitors (e.g., Hg^{+2} , organophosphate, insecticides) on xenobiotic metabolizing enzymes located in small intestine. Changes in enzymes with deficient diet, time of day (diurnal variations), and stress on different xenobiotic metabolizing enzymes in the rabbit intestine will also be studied.

KEYWORD DESCRIPTORS

Drug metabolism; sex differences in extrahepatic drug metabolism; UDP-glucuronyl transferase; activation by detergents in extrahepatic tissues; liver, lung, kidney, intestine, microsomal enzymes; species variations in xenobiotic metabolism; extrahepatic drug metabolism; xenobiotic metabolism by intestinal microsomes; strain variation in drug metabolism; genetic regulation of drug metabolism; hormonal regulation of drug metabolism; genetic regulation of aryl hydrocarbon hydroxylase induction in mice; post-natal development of mixed-function oxidase in intestine; metabolism of aniline in liver from neonate rabbits; aniline metabolism by hemoglobin.

PUBLICATIONS

Chhabra, R. S. and Fouts, J. R.: A comparative study of biochemical properties of mixed-function oxidases (MFO) in liver and intestine of rabbit. Pharmacologist 16: 503, 1974. (Abstract)

Chhabra, R. S. and Fouts, J. R.: Induction of hepatic microsomal drug metabolizing enzymes (HMDME) in a strain of mouse previously believed to be resistant to DDT or 3,4-benzpyrene (BP) treatment. Fifth International Congress on Pharmacology. Abstract-of-Volunteer Papers, p. 242, 1972. (Abstract)

Chhabra, R. S. and Fouts, J. R.: Sex differences in the metabolism of xenobiotics by extrahepatic tissue in rats. Drug Met. Disp. 2: 375-379, 1974.

Chhabra, R. S., Pohl, R. J., and Fouts, J. R.: A comparative study of xenobiotic metabolizing enzymes in liver and intestine of various animal species. Drug Met. Disp. 2: 443-447, 1974.

Chhabra, R. S., Tredger, J. M., Philpot, R. M., and Fouts, J. R.: Selection of inducers: an important factor in characterizing genetic differences to induction of aryl hydrocarbon hydroxylase in strains of mice. Life Sciences 15: 123-130, 1974.

Tredger, J. M., Chhabra, R. S., and Fouts, J. R.: Development of drug metabolizing enzymes in the small intestine and liver of rabbits. Fed. Proc. 34: 3133, 1975. (Abstract)

Project No.: Z01 ES 80005-03 PB

1. Pharmacology Branch
- 2.
3. Research Triangle Park, N.C.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

PROJECT TITLE: "In Vitro Xenobiotic Metabolism by Selected Marine Species and the Effects of Environmental Temperature or Pre-exposure to Environmental Pollutants on These Biotransformation Reactions"

PREVIOUS SERIAL NUMBER: NIEHS-P-006

PRINCIPAL INVESTIGATORS: J. R. Bend, Ph.D., M. O. James, Ph.D.

OTHER INVESTIGATORS: J. R. Fouts, Ph.D., R. M. Philpot, Ph.D., R. J. Pohl, B.S., and N. P. Davidson, B. S.

COOPERATING UNITS: Environmental Biometry Branch, Cornell University,
Mt. Desert Island Biological Laboratory

MAN YEARS

Total:	0.7
Professional:	0.5
Other:	0.2

PROJECT DESCRIPTION

OBJECTIVES: (1) To compare hepatic and extrahepatic microsomal xenobiotic oxidative metabolism in several vertebrate and invertebrate marine species, (2) to characterize the hepatic mixed-function oxidase system of the little skate, Raja erinacea, since it possesses relatively high enzyme activity, (3) to investigate some xenobiotic conjugating enzymes in marine vertebrates and invertebrates, and (4) to study the effects of environmental contaminant administration on in vitro xenobiotic mixed-function oxidation and conjugation by tissues from marine elasmobranchs and teleosts.

METHODS EMPLOYED: Subcellular fractions are prepared from tissue homogenates of freshly collected marine specimens. Standard procedures are utilized for the determination of enzyme activities and quantitation of microsomal cytochromes.

MAJOR FINDINGS: 1. Following pretreatment (orally or IP) with 3-methylcholanthrene (3-MC) or 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), microsomes prepared from little skate (Raja erinacea) liver and kidney had increased benzyrene hydroxylase specific activities in some, but not all,

cases. The lack of response in some individuals may be due to the heterogeneity of the genetic pool of the skates or perhaps the wide variations in absorption of 3-MC and TCDD by the skates. Cytochrome P-448 was not found in skate liver microsomes after either 3-MC or TCDD administration, and there was no increase in the specific P-450 content in treated vs. control skates.

2. The epoxide metabolizing enzymes epoxide hydrase and glutathione (GSH)-S-epoxide transferase were present in liver, kidney, and spiral valve of the Maine marine species tested. Endogenous GSH concentrations were measured in the little skate (Raja erinacea). The three tissues with the most GSH were liver (1.1-3.9 mM), kidney (1 mM), and spiral valve mucosa (0.4-1.8 mM).

3. Hepatic microsomal aniline hydroxylase and benzphetamine demethylase activities of the immature puffin (Fratercula arctica), a sea bird, were significantly increased (3.7-fold and 5.2-fold, respectively) by animal pretreatment with DDE (1,1-dichloro-2,2-bis-[p-chlorophenyl]-ethylene). No induction of either enzyme activity occurred following DDE administration to the white Pekin duck (Anas platyrhynchos).

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The marine ecosystem is contaminated with substantial amounts of pesticides, industrial by-products and other synthetic organic chemicals which are produced in huge quantities by our technologically-orientated society. The ability or inability of marine species, particularly those that are edible or those which are important in the aquatic food chain, to biotransform and excrete these xenobiotics is relevant both to the subsequent fate of these species (in the face of increasing pollution) and to their potential value and danger as direct and indirect foodstuffs for man.

PROPOSED COURSE: 1. Epoxide hydrase and GSH-S-epoxide transferase activities towards epoxides of varying chemical reactivity will be studied to determine the substrate specificities of these two epoxide metabolizing enzymes in hepatic and extrahepatic tissues of several marine vertebrate and invertebrate species, in control animals and in animals previously exposed to environmental pollutants.

2. Using technics which allow the solubilization and separation of the mammalian microsomal mixed-function oxidase system into its three components (cytochrome P-450, lipid, and reductase fractions), we intend to reconstitute this enzyme system from little skate (Raja erinacea) and to identify the heat-sensitive fraction(s). The cytochrome P-450 fractions from control and 3-MC or TCDD pretreated skates will be compared with regard to substrate specificity and ability to form interaction spectra with substrates and inhibitors of the mixed-function oxidase system.

3. In vitro mixed-function oxidation and conjugation rates for several xenobiotics will be measured in marine species acclimated to warm sea water in a Florida laboratory (water temperature ca. 27°C) for comparison to

previous studies on Maine marine species acclimated to cold sea water (12-15°C). Studies with both control and MFO inducer-pretreated animals will be completed.

KEYWORD DESCRIPTORS

In vitro xenobiotic metabolism; vertebrate and invertebrate species; microsomal mixed-function oxygenase system; conjugating enzymes; epoxide hydrase; hepatic and extrahepatic tissues; effects of administration of environmental contaminants; 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD).

PUBLICATIONS

Bend, J. R., Peakall, D. B., Miller, D. S., and Kinter, W. B.: Effects of DDE administration on hepatic microsomal mixed-function oxidase activities in the puffin (Fratercula arctica) and the white Pekin duck (Anas platyrhynchos). Mt. Desert Island Biol. Lab. Bulletin. In press, 1975.

Bend, J. R., Pohl, R. J., Davidson, N. P., and Fouts, J. R.: Response of hepatic and renal microsomal mixed-function oxidases in the little skate, Raja erinacea, to pretreatment with 3-methylcholanthrene or TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin). Mt. Desert Island Biol. Lab Bulletin. In press, 1975.

James, M. O., Fouts, J. R., and Bend, J. R.: In vitro epoxide metabolism in some marine species. Mt. Desert Island Biol. Lab Bulletin. In press, 1975.

Pohl, R. J., Bend, J. R., Guarino, A. M., and Fouts, J. R.: Hepatic microsomal mixed-function oxidase activity of several marine species from Coastal Maine. Drug Metab. Disp. 2: 545-555, 1974.

Project No.: Z01 ES 80006-04 PB
1. Pharmacology Branch
2.
3. Research Triangle Park, N.C.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

PROJECT TITLE: "Environmental Contaminants: Uptake, Distribution, Metabolism, Excretion, and Sites of Storage in Selected Marine Species"

PREVIOUS SERIAL NUMBER: NIEHS-P-007

PRINCIPAL INVESTIGATORS: J. R. Bend, Ph.D., M. O. James, Ph.D., and J. R. Fouts, Ph.D.

OTHER INVESTIGATORS: L. G. Hart, Ph.D., G. L. Foureman, B. S., R. J. Pohl, B. S., and D. P. Rall, M.D., Ph.D.

COOPERATING UNITS: Pharmacokinetics Section, Pharmacology Branch, and Environmental Biometry Branch

MAN YEARS

Total:	0.3
Professional:	0.3
Other:	0.0

PROJECT DESCRIPTION

OBJECTIVES: The objectives of this study are to measure the *in vivo* uptake, distribution, metabolism and excretion of single, purified radiolabeled environmental contaminants, such as certain polychlorinated biphenyl (PCB) isomers, in selected marine species.

METHODS EMPLOYED: At various times after administration (generally parental) of radiolabeled compound, specimens are sacrificed, plasma and tissues are removed, and aliquots are oxidized and/or solubilized prior to liquid scintillation counting. Urinary and biliary excretion rates are also monitored. Metabolites are identified by standard procedures, including glc, mass spectrometry, and NMR.

MAJOR FINDINGS: 1. ^{14}C -Benzoic acid was excreted very slowly in urine following intravascular administration to female dogfish sharks; less than 30% of a 40 mg/kg dose was excreted in 3 days. Residual radioactivity was present primarily in muscle, blood and liver at 48 hrs after treatment. Only traces of urinary radioactivity were present as free benzoic acid, but several metabolites were found. On the other hand,

the herbicide 2,4-D (2,4-dichlorophenoxyacetic acid) was excreted rapidly from dogfish (up to 70% of a 30 mg/kg dose was excreted in 48 hr), and the urinary radioactivity was largely (80-95%) a single polar metabolite which has been tentatively identified as 2,4-dichlorophenoxyacetyltaurine.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Since compounds such as 2,4-D and the PCBs occur widely in nature, the storage, metabolism and elimination of these compounds in animals and man is of importance to environmental health. It is relevant to consider marine species since several pollutants are concentrated by the aquatic food chain and man relies upon several of these for a significant amount of foodstuff. Furthermore, the study of interaction between environmental contaminants, such as PCBs, and other xenobiotics including drugs, food additives, and pesticides with regard to competition for a common storage site in fish and other animals may aid in understanding increased toxicity of pollutant-drug, etc. mixtures or increased tissue storage of pollutants in fish exposed to pollutant mixtures.

PROPOSED COURSE: 1. The uptake, metabolism and elimination of selected radiolabeled aliphatic and aromatic constituents of crude oil (petroleum) will be studied in at least one vertebrate and one invertebrate marine species acclimated to a cold water environment (Maine, 12-15°C) and to a warmer environment (Florida, 25-29°C). The effect of pre-exposure of experimental animals to foreign organic chemicals, such as the PCBs, chlorinated insecticides and hydrocarbons, and to heavy metals on the subsequent uptake, metabolism and elimination of these aliphatic and aromatic radiolabeled crude oil constituents will also be studied in Maine and Florida marine animals.

2. Using xenobiotic conjugates which are excreted in urine or bile, such as ^{14}C -phenacetyl glycine, the effect of environmental temperature on urinary and biliary excretion will be studied in Florida fish that have been acclimated to a variety of temperatures in the laboratory for a prolonged period (2 weeks-1 month) of time.

3. The metabolism and excretion of another herbicide, 2,4,5-T (2,4,5-trichlorophenoxyacetic acid) will be studied in the dogfish shark for comparison to the benzoic acid and 2,4-D disposition data previously reported.

4. Pharmacokinetics of selected pollutants in marine species will be compared with similar data obtained in rats (by the Pharmacokinetics section, Pharmacology Branch) as part of a general program to develop models for pollutant distribution in different species and to be able to extrapolate pollutant distribution data obtained in animals to man.

KEYWORD DESCRIPTORS

Selected marine species; in vivo uptake; distribution; metabolism; excretion; radiolabeled environmental contaminants; ^{14}C -2,4,5,2'5'-pentachlorobiphenyl; ^{14}C -benzoic acid; ^{14}C -2,4-dichlorophenoxyacetic acid (2,4-D).

PUBLICATIONS

Bend, J. R., Hart, L. G., Guarino, A. M., Rall, D. P., and Fouts, J. R.: Distribution and excretion of ^{14}C -2,4,5,2',5'-pentachlorobiphenyl (^{14}C -PCB) in the lobster (Homarus americanus) and the dogfish shark (Squalus acanthias). Pharmacologist 16: 196, 1974. (Abstract)

James, M. O., Bend, J. R., and Fouts, J. R.: Studies on the fate of phenylacetic acid in some fish species. Mt. Desert Island Biol. Lab Bulletin 13: 59-62, 1973.

Project No.: Z01 ES 80007-04 PB
1. Pharmacology Branch
2.
3. Research Triangle Park, N. C.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

PROJECT TITLE: "Hepatic and Extrahepatic Conjugation and Mixed-Function Oxidation Pathways in Mammals and the Effects of Environmental Contaminants and Other Xenobiotics on the Enzymes Involved"

PREVIOUS SERIAL NUMBER: NIEHS-P-003

PRINCIPAL INVESTIGATORS: J. R. Bend, Ph.D., Z. Ben-Zvi, Ph.D., and
M. O. James, Ph.D.

OTHER INVESTIGATORS: J. R. Fouts, Ph.D., F. C. P. Law, Ph.D., T. E.
Eling, Ph.D., R. M. Philpot, Ph.D., G. L. Foureman,
B. S., D. W. Keever, B. S., and J. Van Anda, B. S.

COOPERATING UNITS: Environmental Biometry Branch, Laboratory of Chemistry,
NIAMDD

MAN YEARS

Total:	4.3
Professional:	2.3
Other:	2.0

PROJECT DESCRIPTION

OBJECTIVES: The objectives of this study are (1) the characterization of some microsomal, mitochondrial or soluble conjugating and hydrating enzymes of extrahepatic tissues as compared to the liver enzymes, (2) to determine the effects of selected xenobiotics upon these enzymes, (3) to assess the relative importance in vivo of this extrahepatic enzyme activity as compared to the liver, following ingestion or administration of chemicals and drugs (substrates) via various routes including inhalation, (4) to investigate the perinatal development of some detoxifying enzymes in hepatic and extrahepatic tissues of a few species and to determine the protective role, if any, of these enzymes against localized tissue toxicity induced by chemically reactive electrophilic metabolites, such as epoxides, (5) to compare the metabolism of chemically reactive epoxides and their unsaturated hydrocarbon precursors in an isolated perfused rabbit lung preparation, and (6) to assess the role of the cell's nucleus (nuclear membrane and

nucleoplasm) in the formation and subsequent biotransformation of chemically reactive metabolites.

METHODS EMPLOYED: Differential centrifugation, spectroscopic, fluorometric and radiochemical analyses.

MAJOR FINDINGS: 1. The mitochondrial enzyme glycine N-acyltransferase was characterized in liver and kidney of rabbit and rat using ^{14}C -phenacetyl coenzyme A as the acyl donor. Specific activity was about 25 times larger in rabbit kidney than in liver. Other rabbit organs tested had undetectable (lung) or extremely low (intestine) activity. Total kidney enzyme activity was about 10 times that of whole liver in the rabbit. In rats, renal specific activity was 3 times that of liver, but total conjugating activity of the two organs was similar.

In young rabbits, hepatic glycine N-acylase attained adult specific activities by 3-4 weeks of age as compared to 15 weeks of age for renal enzyme activity. In rat liver, adult enzyme activities were achieved by 1 week of age.

2. Styrene oxide - ^{14}C -8 (STO) was metabolized quite rapidly by an isolated perfused rabbit lung preparation (IPL) to four water-soluble metabolites and one nonpolar metabolite. The nonpolar metabolite was phenylethane-1,2-diol, which is formed from STO by microsomal epoxide hydrase. The major water-soluble (polar) metabolite was the glutathione (GSH) conjugate of STO, formed by soluble fraction GSH-S-epoxide transferase. A second quantitatively important polar metabolite appears to be the cysteinylglycine derivative resulting from γ -glutamyltranspeptidase activity on the GSH conjugate. The other 2 water-soluble metabolites are very minor and have not been identified. The amount of STO metabolized after 30 min by the IPL is concentration dependent over the range of 10 μmol s-70 μmol s/lung (200 ml circulating perfusate). Higher STO concentrations are toxic in the lung. Quantitatively, the polar metabolites are substantially more important than the nonpolar diol. We have also demonstrated depletion (50%) of GSH in the lung following prolonged perfusion (60 min) with high but still non-toxic levels of STO (66 μmol s)

3. The rate of metabolism of ^{14}C -styrene oxide by 10,000 x g preparations of rabbit liver, lung and gut was compared to the rate of STO metabolism by microsomes vs. soluble fractions (microsomal supernatant). In the presence of soluble fraction, the only major water-soluble metabolite of STO formed was the GSH conjugate, whereas in 10,000 x g preparations there were also considerable amounts of another polar conjugate, presumably the cysteinylglycine derivative. This latter conjugate represents the second step in mercapturic acid biosynthesis.

4. The response to enzyme inducers of hepatic and extrahepatic (lung, kidney, intestine and testis) microsomal epoxide hydrase and soluble

fraction GSH-S-epoxide transferase (using STO as substrate) has been studied in the rat following administration of phenobarbital (80 mg/kg IP for 3 days, killed 24 hr after last dose), TCDD (10 μ g/kg IP 5 days before sacrifice), 1,2,3,4-dibenzanthracene (80 mg/kg IP for 3 days, killed 24 hr after last dose) or pregnenolone-16 α -carbonitrile (20 mg/kg IP for 2 days, killed 24 hr after last dose). The most dramatic increases in epoxide-metabolizing enzyme activity occurred in liver after phenobarbital; hydase activity was induced 2.5-fold and transferase activity 1.8-fold. Although pulmonary transferase activity of these animals tended to be slightly increased (about 1.3-fold), there was no alteration in lung epoxide hydase activity. In general, the results obtained demonstrated a much lower response (vs. liver) of extrahepatic epoxide hydase and GSH-S-epoxide transferase to administration of known inducers of the microsomal xenobiotic-metabolizing enzyme system in this species.

5. Age-related development of GSH-S-epoxide transferase and epoxide hydase was monitored in hepatic and extrahepatic (placenta, lung, gut, kidney) tissues of the New Zealand rabbit. At the earliest gestational age studied (20 days), lung (60% of adult specific activity), gut (80%) and kidney (50%), all contained higher specific GSH-S-epoxide transferase activity than did liver (4% adult activity). For the three extrahepatic enzymes, a slight decrease of activity was observed just after birth (at 1 day of age), which had disappeared by 3 days of age; no such decrease was observed in liver. Maturation of epoxide transferase activities to adult levels occurred more slowly in liver and lung (between 1 and 2 months of age) than in gut (1 month) or kidney (13 days). Placenta contained transferase activity which decreased with gestational age. Renal and pulmonary epoxide hydase were detectable, but very low, at birth. Hepatic hydase activity was low during gestation and for the first 3 weeks of life. However, there was a rapid increase in activity between 20 and 30 days of age, by which time activities could not be distinguished from those of adults.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The conjugation of xenobiotics or their metabolites which are chemically reactive (e.g., epoxides and N-oxides) by liver, lung, gut and other extrahepatic tissues may play a significant role in the overall toxicity of agents which enter the body via the pulmonary system or the alimentary canal. The developmental patterns of conjugation pathways active in detoxication are of importance since these may determine the susceptibility of the fetus and neonate to environmental contaminants, and may also be related to chemical carcinogenesis and teratogenesis. In the real-life situation one is very rarely exposed to a single xenobiotic; rather one comes in contact with a large number of synthetic organic chemicals including food additives, drugs, plasticizers, insecticides, etc. For this reason investigations concerning interactions between more than one foreign compound are of relevance to environmental health.

PROPOSED COURSE: 1. The study of ^{14}C -styrene oxide (STO) metabolism by the isolated perfused lung (IPL) will be completed, and the ratio of phenylethyldiol metabolite (formed by epoxide hydrase) to water-soluble thioether metabolites (formed by epoxide transferase) will be compared to the ratio of these same two metabolites resulting when ^{14}C -styrene is metabolized by cell fractions obtained from lung homogenates (microsomes and soluble fraction). Styrene metabolism by the IPL following tracheal intubation (as opposed to adding styrene oxide to the perfusion fluid) will also be investigated, as will the effects of exposure of rabbits to environmental contaminants such as O_3 , SO_x and NO_2 upon the subsequent metabolism of STO by the IPL.

2. The metabolites resulting from perfusion of ^{14}C -benzo[a]pyrene through the rabbit IPL and also from *in vitro* liver and lung microsomal preparations will be identified by high pressure liquid chromatographic analysis and comparison to synthetic reference standards. Subsequently, we will study the biotransformation of radiolabeled K-region and non-K-region polycyclic aromatic hydrocarbons by the IPL. This work will be done in collaboration with Dr. D. Jerina of the Laboratory of Chemistry, NIAIMDD.

3. The substrate specificity of GSH-S-epoxide transferase will be studied in soluble fractions (microsomal supernatant fractions) of liver, lung, gut and kidney of rabbit and possibly other selected species using a number of different radiolabeled K-region and non-K-region epoxides. Similar studies will be done with fetal, neonatal and adult enzymes of one species, probably rabbit, and attempts will be made to correlate covalent binding of certain chemically reactive epoxides in neonatal and fetal tissues to the activities of the epoxide detoxifying enzymes in the various tissues (i.e., epoxide hydrase and GSH-S-epoxide transferase).

4. Studies comparing the induction of hepatic and extrahepatic epoxide hydrase and epoxide transferase will be completed in the rat and rabbit.

KEYWORD DESCRIPTORS

Hepatic and extrahepatic enzymes; mammals; mixed-function oxygenases; xenobiotic conjugating enzymes; isolated perfused lung; epoxide metabolism; microsomes; mitochondria and microsomal supernatant (soluble) enzymes; perinatal development; effects of environmental contaminants on enzymes involved.

PUBLICATIONS

Bend, J. R. and Hook, G. E. R.: Hepatic and extrahepatic mixed-function oxidases. In Lee, D. H. K. and Murphy, S. D. (Ed.): Handbook of Physiology, Section on Environmental Physiology. American Physiological Society, in press, 1975.

Bend, J. R., James, M. O., Devereux, T. R., and Fouts, J. R.: Toxication-detoxication systems in hepatic and extrahepatic tissues in the perinatal period. Proceedings of the International Symposium on Perinatal Pharmacology, Raven Press, New York, in press, 1975.

Bend, J. R., James, M. O., and Fouts, J. R.: Perinatal development of glutathione S-epoxidetransferase (ETase) and epoxide hydrase (EH) in liver and selected extrahepatic tissues of the guinea pig. Fed. Procs., Vol. 33 (3), p. 566, 1974. (Abstract)

Fouts, J. R., Bend, J. R., James, M. O., and Devereux, T. R.: Perinatal development of N-oxidase, N-demethylase, epoxide hydrase, and glutathione (GSH)-S-epoxide transferase in hepatic and extrahepatic tissues of guinea pigs and rabbits. International Symposium on Perinatal Pharmacology, Milan, Italy, June 13-15, 1974. (Abstract)

James, M. O. and Bend, J. R.: Perinatal development of glycine N-acyltransferase (GNAT) in rabbit kidney and liver. Fed. Proc., 34, 812, 1975. (Abstract)

James, M. O., Fouts, J. R., and Bend, J. R.: Hepatic and extrahepatic in vitro metabolism of an epoxide (8-¹⁴C-styrene oxide) in the rabbit. Biochem. Pharmacol. in press, 1975.

Law, F. C. P., Eling, T. E., Bend, J. R., and Fouts, J. R.: Metabolism of xenobiotics by the isolated perfused lung: comparison with in vitro incubations. Drug Metab. Disp. 2: 433-442, 1974.

Project No.: Z01 ES 80008-01 PB

1. Pharmacology Branch
- 2.
3. Research Triangle Park, N. C.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

PROJECT TITLE: "Studies on the Transport and Metabolism of Prostaglandins (PG) in Lung"

PREVIOUS SERIAL NUMBER: None

PRINCIPAL INVESTIGATORS: T. E. Eling, Ph.D. and M. W. Anderson Ph.D. (Environmental Biometry Branch)

OTHER INVESTIGATORS: H. Hawkins, B. S., J. Guthrie, B. S., E. Postlethwaite, B. S., and R. J. Sensenig, B. S. (Environmental Biometry Branch)

COOPERATING UNIT: Environmental Biology and Chemistry Branch, Aerotoxicology Section

MAN YEARS

Total:	1.95
Professional:	0.70
Other:	1.25

PROJECT DESCRIPTION

OBJECTIVES: (1) To elucidate the mechanism(s) for the transport of PGs across cell membranes, (2) to determine the effects on this transport system of various anti-inflammatory agents and exposures of animals to pollutants, and (3) to relate alteration in this PG-transport system to physiological effects of pollutants on the lung.

METHODS EMPLOYED: An isolated perfused rat lung was used to examine the uptake, metabolism, and efflux of PGs and their metabolites from lung tissue. The isolated perfused lung was designed to permit infusion of a constant concentration of PGs and perfusion with drug-free perfusate. PG metabolites were isolated from the perfusate by extraction and separated by TLC techniques.

MAJOR FINDINGS: ^3H -PGF₂ was accumulated into lung tissue by a carrier-mediated process. Accumulated $^2\alpha$ ^3H -PGF₂ was metabolized to either 15-keto-PGF₂ or 15-keto-dehydro PGF₂. The accumulation of PGF₂ and the subsequent efflux of PGF₂ and its metabolites were very rapid. The transport process for PGs in rat lung was specific; PGF₂ and PGE₁ were accumulated in lung while PGF₁ and PGA₁ were not accumulated in lung tissue. The metabolite 15-keto-

PGF_{2α} was not taken up by the lung but when formed inside the cell from PGF_{2α} it was rapidly effluxed from the lung. This indicates that lung cell membranes may be asymmetric with respect to PG passage. The transport of PGs appears to be related to the rate at which PGs are supplied to the lung rather than to the concentration in the perfusate. Supply rate (perfusate concentration x flow rate) should be used in species to species comparisons of transport systems in isolated organ systems.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: PGs have a large diversity of physiological effects. Alterations in PG control of cellular events may be related to transport of PGs across cell membranes. The lung is an important site for the synthesis and metabolism of PGs; alterations in the PG transport and metabolism systems may be related to toxic effects of exposures to pollutants.

PROPOSED COURSE: (1) To study the effect of anti-inflammatory drugs on PG transport in the lung, (2) to examine the effect of exposure of rats to airborne pollutants on PG detoxification systems, (3) to use isolated cell systems to examine the mechanisms for the transport of PGs into and out of cells and apply the information obtained from this study to the intact lung system, and (4) to relate PG transport in cells to cellular levels of cyclic AMP-GMP and cell proliferation.

KEYWORD DESCRIPTORS

Prostaglandins; non-respiratory lung function; prostaglandin transport; effect of pollutants on prostaglandin transport system; prostaglandin metabolism by lung; isolated perfused lung; pharmacokinetics of prostaglandin disposition.

1. Pharmacology Branch
- 2.
3. Research Triangle Park, N. C.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

PROJECT TITLE: "Studies on the Mechanisms of Accumulation of Drugs and Pollutants in the Lung: Investigation of the Transport of Substances from Pulmonary Circulation and Inhaled Air into Lung Tissue and of Metabolism of Xenobiotics by the Isolated Perfused Lung. Use of the Isolated Perfused Lung to Examine the Effect of Pollutants on Lung Functions"

PREVIOUS SERIAL NUMBER: NIEHS-P-010

PRINCIPAL INVESTIGATORS: T. E. Eling, Ph.D. and M. W. Anderson, Ph.D.
(Environmental Biometry Branch)

OTHER INVESTIGATORS: R. D. Pickett, Ph.D., F. C. Law, Ph.D., H. Hawkins, B. S.,
J. Guthrie, B. S., and A. W. Wilson, Ph.D. and R. J.
Sensenig, B. S. (Environmental Biometry Branch)

COOPERATING UNITS: Environmental Biometry Branch, Environmental Biology and
Chemistry Branch (Histology, Electron Microscopy)

MAN YEARS

Total:	2.6
Professional:	2.0
Other:	0.6

PROJECT DESCRIPTION

PART I: "Studies on the Mechanisms of Accumulation and Metabolism of Chemicals and Pollutants by the Lung"

OBJECTIVES: Study the processes and mechanisms for uptake and accumulation of basic chemicals (e.g., amines) in lung tissue. Assess the role of the lung in the disposition of chemicals and develop pharmacokinetic models for disposition of chemicals in the lung.

METHODS EMPLOYED: The lung perfusion apparatus consists of an artificial thorax, a recirculating blood supply, and an air supply. The lungs are ventilated with 95% air - 5% CO₂ by applying an alternating negative pressure to the artificial thorax. The circulatory system was designed with a blood reservoir directly above the lungs to which the labeled chemicals were added and from which samples of blood were removed for analysis. The apparatus was

modified so that the rates of uptake and the rates of efflux of chemicals from lung could be examined.

Metabolites of the compounds as found in the blood and lung were studied by thin-layer, paper and gas liquid chromatographic techniques. Viability of the perfused lung was confirmed by physiologic parameters as well as light and electron microscopy. Blood pH was also monitored over the perfusion period.

MAJOR FINDINGS: We have developed detailed models for the dynamic interaction of the perfused lung and circulating basic amines including amphetamine (AMPH), imipramine (IMIP), and methadone (MET). All of these compounds rapidly diffuse into and out of the lung's vascular space. Little AMPH was bound in the intracellular space while significant amounts of IMIP and MET partitioned to the intracellular space. AMPH was accumulated into the cells by a carrier-mediated transport process. IMIP and MET were accumulated by processes of diffusion and binding. Approximately 10-15% of accumulated MET was metabolized by the MFO system. AMPH did not persist in the lung and effluxed very rapidly from the tissue once influx of AMPH stopped. IMIP and MET effluxed with a half-life of 8 minutes. Approximately 20-30% of accumulated IMIP and MET was not effluxable. This non-effluxable pool is responsible for the persistence of basic amines in lung tissue which occurs after blood levels of the chemicals become zero. The non-effluxable pools for IMIP and MET were not the result of covalent binding of IMIP and MET but appeared to be the result of compartmentalization of these chemicals. The non-effluxable pool for certain amines may be associated with phospholipid-chemical complexes in concentric lamellar bodies (CLB) and may be involved in pulmonary phospholipidosis produced by a variety of basic amines (e.g., chlorphentermine).

We have also studied the metabolism of several chemicals by the isolated perfused rabbit lung. Of the chemicals studied, only parathion, methadone, and pentobarbital were significantly degraded by isolated perfused rabbit lung. However, parathion, methadone, pentobarbital, imipramine, amphetamine, and chlorcyclizine were all degraded by lung and liver homogenates and sub-cellular fractions. Methadone was metabolized to two cyclic metabolites ($M_1 - M_2$) by both lung homogenates and the isolated perfused lung. However, the ratio of M_1 to M_2 obtained with lung homogenates was different from that obtained in isolated perfused lung. Thus, extrapolation of metabolism data from in vitro incubation mixture to intact lung may not always be possible.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Development of a method for the perfusion of an isolated intact lung allows studies with the lung uncomplicated by other organ functions. This preparation is perfused and ventilated similarly to an intact organ and thus good correlation of in vivo and in vitro studies is likely.

The lung can act as an important site for accumulation of xenobiotics and this accumulation could result in detoxification or altered toxicity. In addition, those xenobiotics which accumulate in the lung could affect the accumulation and storage of other exogenous compounds or of endogenous substances such as norepinephrine and 5-hydroxytryptamine. The elucidation of the mechanism of accumulation could afford a means for predicting possible toxic effect and

interactions of one chemical with another.

Examination of the non-effluxable pool can permit development of methods for predicting or affecting chemical-induced lipidosis in lung tissue.

PROPOSED COURSE: (1) To examine the efflux characteristics of phentermine and chlorphentermine in the isolated perfused rabbit lung and to relate the presence of a non-effluxable pool of a chemical or drug to the production of lung phospholipidosis by these chemicals, (2) investigate in more detail the effects of the non-nitrogenous portion of amines on persistence in the lung, (3) study the interaction between endogenous and exogenous amines at their sites of lung uptake and release, (4) develop pharmacokinetic models for the accumulation of amines in lung tissue, and (5) assess the role of lung phospholipid and CLB in the accumulation and persistence of basic amines.

PART II: "Studies on the Uptake and Metabolism of Endogenous Substances by the Lung"

OBJECTIVES: To study the uptake and metabolism of endogenous substances, such as 5-hydroxytryptamine, norepinephrine, prostaglandins and angiotensin by lung. Examine the interactions between the accumulation of endogenous and exogenous substances by lung and study the effect of inhaled environmental agents on such non-respiratory functions of the lung.

METHODS EMPLOYED: See "Methods Employed" in Part I.

MAJOR FINDINGS: Serotonin (5-HT) is removed from the venous circulation in lung by a uni-directional transport system which is Na⁺ dependent. 5-HT is metabolized by the intracellular monoamine oxidase to 5-hydroxyindole acetic acid and this metabolite escapes by diffusion back into the arterial circulation. We have shown that the components of this detoxification system for 5-HT can be separately inhibited. For example, we have inhibited the metabolism step without affecting the transport system by using the monoamine oxidase inhibitor, pargyline. The 5-HT which was transported into the cell returned unaltered to the circulation by diffusion. Conversely, imipramine inhibits the transport system without affecting the metabolism.

A pharmacokinetic model was developed for the 5-HT detoxification system. From this model it was evident that flow rates are important in examining transport systems in isolated organs. Thus, development of a pharmacokinetic model has identified potential erroneous experimental designs in studying transport systems in perfused organs.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The lung is an important site for the detoxification of several endogenous compounds. Understanding the mechanism for these detoxifications, etc., could aid in understanding lung function and the effect of certain environmental insults on the lung.

Elucidation of the mechanism of lung uptake and metabolism of chemicals might allow prediction of possible toxic interactions between endogenous and exogenous substances.

PROPOSED COURSE: We will examine the effect of exposure to pollutants on the 5-HT detoxification system of the lung.

KEYWORD DESCRIPTORS

Pharmacokinetics; non-respiratory lung functions; chemical- and drug-induced phospholipidosis; imipramine; methadone; 5-HT; amphetamine; chemical- and drug-binding to phospholipid in lung; effect of pollutants on non-respiratory lung function; isolated perfused lung; chemical or drug metabolism by isolated perfused lung; parathion; pentobarbital; chlorcyclizine.

PUBLICATIONS

Anderson, M. W., Eling, T. E., and Philpot, R. M.: Metabolic functions of lung. In Lenfant, C. (Ed.): Monograph 5 of the series entitled "Lung Biology in Health and Diseases." Steroids and Drugs, Chapter 5. New York, N. Y., Marcel Dekker, Inc. In press.

Eling, T. E., Pickett, R. P., Orton, T. C., and Anderson, M. W.: A study of the dynamics of imipramine accumulation in the isolated perfused rabbit lung. Drug Metab. Disp. In press.

Law, F. C. P., Eling, T. E., Bend, J. R., and Fouts, J. R.: Metabolism of xenobiotics by isolated perfused lung. Comparison with in vitro incubation. Drug Metab. Disp. 2: 433-442, 1974.

Pickett, R. P., Anderson, M. W., Orton, T. C., and Eling, T. E.: The pharmacodynamics of 5-hydroxytryptamine uptake and metabolism by isolated perfused rabbit lung. J. Pharmacol. Exp. Ther. In press.

Project No.: Z01 ES 80010-04 PB

1. Pharmacology Branch
- 2.
3. Research Triangle Park, N. C.

PHS-NIH

Individual Project Report

July 1, 1974 through June 30, 1975

PROJECT TITLE: "Prostaglandin Biosynthesis and Metabolism by Lungs. Effect of Pollutants on This System"

PREVIOUS SERIAL NUMBER: NIEHS-P-023

PRINCIPAL INVESTIGATORS: T. E. Eling, Ph.D. and M. W. Anderson, Ph.D.
(Environmental Biometry Branch)

OTHER INVESTIGATORS: J. Guthrie, B. S., H. Hawkins, B. S., and J. Sensenig,
B. S. (Environmental Biometry Branch)

COOPERATING UNITS: Aerotoxicology Section, Pharmacology Branch and
Environmental Biology and Chemistry Branch

MAN YEARS

Total:	0.7
Professional:	0.3
Other:	0.4

PROJECT DESCRIPTION

OBJECTIVES: This is a continuing project directed toward obtaining a better understanding of lung function. The primary objective is to investigate the biosynthesis and metabolism of prostaglandins by the lung and to relate changes in steady-state concentrations of the various prostaglandins to biological effects. In addition, the effects of various environmental agents (SO_2 , O_3 , NO_2 , O_2 , etc.) on the ability of the lung to synthesize and degrade prostaglandins will be investigated. An attempt will be made to correlate the biological effect of pollutants and modulation of prostaglandin levels.

METHODS EMPLOYED: Prostaglandins are analyzed by a combination of quantitative thin-layer chromatography and liquid scintillation counting. The radioactive prostaglandins, produced by lung tissue from the precursor ^{14}C -arachidonic acid, are extracted from the incubation medium and separated on thin-layer plates. The radioactive areas are measured by liquid scintillation techniques. Guinea pig or rat lung serves as an enzyme and cofactor source for both biosynthesis and metabolism. Further identification of the prostaglandins uses a combination of thin-layer

chromatography, continuous gradient elution column chromatography and mass spectrometry. Prostaglandin metabolism was estimated by incubation of $^3\text{H-PGF}_2$ with the enzyme source, extraction from the incubation mixtures and separation of nonmetabolized $^3\text{H-PGF}_2$ from metabolites. Metabolism was measured by the disappearance of $^3\text{H-PGF}_2$.

MAJOR FINDINGS: Lung tissue biosynthesizes and degrades prostaglandins (PG). The PG synthesizing system in guinea pig lung converts ^{14}C -arachidonic acid to PGF_2 and an unknown PG-like material. An assay for the enzyme system was developed, characterized and optimized. We have established the chemical identity of PGF_2 produced by our enzyme system with GLC-mass spectrometry. The unknown has been identified by mass-spectrometric techniques as 8-(1-hydroxy-3-oxopropyl)-9,12-dihydroxy-5,10-heptadecadienoic acid. This compound has recently been isolated from guinea pig lung incubation mixtures and designated PHD. From our studies and those of other workers, it is apparent that guinea pig lung does not biosynthesize large amounts of PGE_2 and PGF_2 but produces large amounts of other "prostaglandin-like" compounds. These "prostaglandin-like" compounds are produced by conversion of an unstable bioactive cyclic intermediate in prostaglandin biosynthesis. The bioactivities of PHD and the other products from the cyclic intermediate have not been assessed.

PGs are metabolized by guinea pig and rat lung dehydrogenases and reductases. Using $^3\text{H-PGF}_2$ as substrate, an in vitro assay system was developed and the metabolites isolated and identified by combined GLC-mass spectrometry.

The effect of environmental agents on the rates of biosynthesis and metabolism in vitro were investigated. We have established that exposure of guinea pig to SO_2 at various concentrations did not alter PG biosynthesis and metabolism. However, exposure of guinea pigs to 100% O_2 depressed PG metabolism but did not alter biosynthesis. Preliminary experiments indicated that exposure of rats to H_2SO_4 mist inhibited PG metabolism while exposure of rats to O_2 did not alter PG degradation. We are currently investigating these effects³ in more detail.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Prostaglandins represent a class of endogenous substances which possess unique biological activity in lung. Prostaglandins of the E class are potent bronchial dilators, while prostaglandins of the F class are potent constrictors. Both classes of prostaglandins have been implicated in inflammatory responses. Because these substances are not stored in most tissues, control of biosynthesis and metabolism is important to their physiological activity. Pathological changes or conditions produced in various disease states or in response to pollutants or environmental agents could involve an effect on either prostaglandin biosynthesis or metabolism. Understanding the mechanisms for the control of prostaglandin levels in lung tissues should contribute to the overall understanding of biological functions of these lipids.

Depression of the lung-metabolizing system could be of direct medical importance because prostaglandins and their derivatives have been used as therapeutic agents. Thus, interactions are possible and should be studied.

PROPOSED COURSE: (1) Investigate the effect of animal exposure to oxidants (O_3 , NO , NO_2) on the PG-metabolizing system of guinea pig and rat lung, (2) establish time course and dose-response to oxidants (O_3 , NO_2) on the PG metabolism, (3) study the mechanism of inhibition of metabolism. Determine if this inhibition is a specific effect related to oxidant toxicity to lung, or is a nonspecific effect, (4) determine the pathologic and/or physiologic effects of increased PG in lung, (5) investigate effects of respiratory diseases or allergic conditions on biosynthesis and metabolism of PG in lung, (6) perform comparative metabolism studies using isolated perfused lung preparations (comparison with microsomal systems), and (7) investigate PG metabolism and oxidant effects on this metabolism in various animal species.

KEYWORD DESCRIPTORS

Prostaglandins; prostaglandin biosynthesis and metabolism; cyclic intermediate; sulfuric acid mist; SO_2 ; NO_2 ; O_3 ; lung cell fractions.

PUBLICATIONS

Parkes, D. G. and Eling, T. E.: The influence of environmental agents on prostaglandin biosynthesis and metabolism. Inhibition of 15-hydroxyprostaglandin dehydrogenase by exposure of guinea pigs to 100% oxygen at atmospheric pressure. Biochem. J. 146: 549-556, 1975.

Project No.: Z01 ES 80011-02 PB

1. Pharmacology Branch

2.

3. Research Triangle Park, N. C.

PHS-NIH

Individual Project Report

July 1, 1974 through June 30, 1975

PROJECT TITLE: "Disposition of 2,4,5,2',5'-Pentachlorobiphenyl (PCB) and Indocyanine Green (ICG) in Biliary Fistula Rats and in Isolated Perfused Rat Livers"

PREVIOUS SERIAL NUMBER: NIEHS-P-012

PRINCIPAL INVESTIGATOR: S. W. Hwang, Ph.D. and L. G. Hart, Ph.D.

OTHER INVESTIGATOR: M. Clemons

COOPERATING UNIT: Environmental Biometry Branch

MAN YEARS

Total:	1.0
Professional:	0.8
Other:	0.2

PROJECT DESCRIPTION

OBJECTIVES: Polychlorinated biphenyls (PCBs) are water-insoluble synthetic chemicals which are widely used in industrial applications and which have been shown to be widespread environmental contaminants. This investigation is part of the research program designed to study comprehensively the pharmacokinetics of disposition and metabolism of 2,4,5,2',5'-pentachlorobiphenyl (PCB) in the rat. The main purpose of our study was to further characterize the uptake and excretion of PCB in the isolated perfused rat liver preparation and compare these parameters with those for a water-soluble model compound, indocyanine green (ICG). The role of different types of perfusion media on hepatic disposition of PCB and ICG was evaluated.

METHODS EMPLOYED: The perfusion of liver was performed in a Miller-type liver perfusion apparatus. Different perfusion media were used: (1) Krebs-Ringer carbonate buffer containing 5 mM glucose and 4.5% bovine serum albumin, (2) heparinized rat blood diluted three times with medium 1, (3) washed bovine erythrocytes diluted with medium 1 to give a hematocrit of 0.35, (4) heparinized whole rat blood. Male rats, $\approx$ 325 g, were used as the liver donor. After pentobarbital anesthesia, the bile

duct and portal vein were cannulated. The inferior vena cava was tied below the liver and a cannula was inserted in the vena cava above the diaphragm. The isolated liver was then connected to the liver perfusion apparatus with the portal vein cannula. The perfusion medium was pre-heated to 38° C and gassed with O₂-CO₂ (95-5%). Perfusion pressure was kept constant at 18 cm. Flow rate of the medium through liver was measured with an extracorporeal flowmeter. Radioactivity of PCB in bile and media were measured as previously described. ICG was measured spectrophotometrically.

MAJOR FINDINGS: 1. The characteristics and kinetics of the uptake by the liver and excretion into the bile of PCB administered to bile fistula rats were reported last year, as were the initial results of studies using the isolated perfused rat liver (IPL).

2. Using an artificial perfusate (Krebs-Ringer carbonate buffer containing 5 mM glucose and 4.5% bovine serum albumin), we studied the effects of alterations in standard perfusion conditions on the hepatic disposition of PCB, and the model compound ICG. Alkaline pH (9.5) or lowered perfusate temperature (25°) resulted in a decreased disappearance rate and decreased liver concentration of both PCB and ICG, while gassing the perfusate with N₂ instead of O₂-CO₂ resulted in a decreased disappearance rate for ICG but had little effect on rate of disappearance of PCB. Acidic pH (5.4) decreased the biliary excretion rate for PCB, but had no effect on the disappearance rate or hepatic concentration of either compound. These results are consistent with the facts that ICG and probably PCB are, in part, actively transferred from plasma to bile, while PCB also requires enzymatic metabolism prior to being excreted in the bile.

3. Pretreatment of liver donor rats with phenobarbital (PB) resulted in increased disappearance of ICG from the perfusion medium and increased concentrations of ICG in perfused livers from PB-treated rats but had no effect on the hepatic disposition of PCB.

4. When livers were perfused with different concentrations of ICG (3.2, 8 and 16 mg/100 ml perfusate) and then effluxed with fresh medium, there appeared to be two separate liver fractions containing ICG, one which was effluxable and one which was not. Unchanged PCB was rapidly effluxed from the liver while its more polar metabolites were effluxed via a bi-exponential process.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE:

Studies of PCB disposition in animal systems may help us to understand how this widely used class of chemicals may be hazardous to the health of animals and humans. Studies using the isolated perfused liver system may contribute to clarifying the differences in hepatic handling of compounds with different lipid solubilities, each as PCB and ICG. Since many environmental chemicals are primarily handled by the liver, the perfused liver system can be an important tool for in-depth studies on the

disposition of these chemicals. Studies on isolated perfused organs are useful in constructing pharmacokinetic models for chemical disposition in the intact animals. These models can then be used in extrapolating chemical disposition from one species to another.

PROPOSED COURSE: Part of this study has been completed and the remainder will be incorporated into a related ongoing project.

KEYWORD DESCRIPTORS

Isolated perfused rat liver; pentachlorobiphenyl; indocyanine green; effects of pH, temperature and N_2 on chemical disposition by liver; biliary excretion of chemicals; hepatic uptake of chemicals; effects of phenobarbital on hepatic disposition of chemicals; pharmacokinetics.

Project No.: Z01 ES 80012-01 PB
1. Pharmacology Branch
2.
3. Research Triangle Park, N. C.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

PROJECT TITLE: "Uptake, Metabolism, and Disposition of Polychlorinated Biphenyls (PCBs) by the Isolated Perfused Rat Liver"

PREVIOUS SERIAL NUMBER: None

PRINCIPAL INVESTIGATOR: H. M. Mehendale, Ph.D.

OTHER INVESTIGATORS: H. B. Matthews, Ph.D., T. E. Eling, Ph.D., M. W. Anderson, Ph.D., S. Vicinus, B. S.

Cooperating Unit: Environmental Biometry Branch

MAN YEARS

Total:	1.2
Professional:	0.5
Other:	0.7

PROJECT DESCRIPTION

OBJECTIVES: We developed an isolated perfused rat liver preparation (IPL) suitable for pharmacokinetic studies with xenobiotics. This IPL was used to study uptake, metabolism, accumulation, and excretion of PCBs. We used an homologous series of PCBs (4-chlorobiphenyl, I; 4,4'-dichlorobiphenyl, II; 2,4,5,2',5'-pentachlorobiphenyl, III; and 2,4,5,2',4',5'-hexachlorobiphenyl, IV) to investigate the relationship of degree of chlorination of PCBs to their uptake, metabolism, and excretion by the liver preparations. These studies were directed towards determining the role of the liver in the overall whole animal disposition of PCBs.

METHODS EMPLOYED: The liver perfusion apparatus developed for this work was a modified version of the Miller apparatus. Rat liver was surgically removed after cannulating the bile duct, portal vein and the thoracic vena cava. The inferior vena cava was ligated. The liver was perfused with 30% rat blood in Krebs-Ringer buffer solution (pH 7.4) containing 5 mM bovine serum albumin and 4.5 mM glucose. Oxygenated blood entered through the portal vein and effluxed via the thoracic vena cava cannula. These experiments were conducted using

a recirculating perfusate. Labeled PCB's were introduced into the perfusate at zero time after the liver had equilibrated for 30 min. Bile, perfusate and liver homogenate samples were assayed for radioactivity. Liver homogenate, perfusate, and bile samples were also extracted and the total radioactivity divided into parent compound and metabolites using conventional extraction and TLC methods.

MAJOR FINDINGS: An isolated perfused rat liver preparation suitable for pharmacokinetic studies was developed and is being used to study the disposition of PCB homologs by the rat liver. Viability of the preparation was ascertained by a combination of tests including visual examination, glucose and oxygen utilization by the liver, and bile flow as well as histological examination of the liver after varying periods of perfusion up to 5 hours. All four PCB homologs were rapidly taken up by the liver from a recirculating perfusate and after the initial 30 minutes, a steady-state was established with respect to the total radioactivity in the perfusate. The rate of biliary excretion was greatest with compound I, followed by II, III, and IV, in that order, indicating an inverse relationship with the degree of chlorination. Biliary excretion and hepatic uptake were linear for 1,3, and 10 nmole PCB/ml perfusate of compounds I and IV. The rate of biliary excretion of compound I was exponentially related to its concentration in the liver. A similar relationship existed for compound II. However, biliary excretion of compounds III and IV was not related to hepatic concentration. The concentration of the unchanged I in the perfusate declined biexponentially corresponding to a 2-compartment model: the initial rapid phase of uptake by the liver ($t_{1/2} = 7$ min) was followed by a second slow phase of uptake ($t_{1/2} = 270$ min).²

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Due to the widespread occurrence of PCBs in the environment and in all the biological species examined including man, there is increasing concern about the effects of these chlorinated compounds on human health. These studies are a part of efforts to construct a pharmacokinetic model for the uptake and disposition of PCB's in animals. This particular study is directed towards understanding and quantifying the role of the liver in the whole body disposition of PCB's.

PROPOSED COURSE: The studies carried out with compound I will be extended to compounds II, III, and IV in order to investigate further the relationship of degree of chlorination to the hepatic uptake, metabolism, and biliary excretion of these PCBs. Studies directed at understanding the relationship between metabolism and biliary excretion will be undertaken.

KEYWORD DESCRIPTORS

Hepatic disposition; polychlorinated biphenyl (PCBs); isolated perfused rat liver; degree of chlorination correlated with bile excretion; pharmacokinetic models of PCB distribution; bile excretion of PCBs; PCB metabolism in relation to bile excretion.

1. Pharmacology Branch
- 2.
3. Research Triangle Park, N. C.

PHS-NIH

Individual Project Report
July 1, 1974 through June 30, 1975

PROJECT TITLE: "Uptake, Metabolism, and Disposition of Aldrin and Dieldrin by the Isolated Perfused Rabbit Lung"

PREVIOUS SERIAL NUMBER: NIEHS-P-012

PRINCIPAL INVESTIGATOR: H. M. Mehendale, Ph.D.

OTHER INVESTIGATORS: E. A. El-Bassiouni, Ph.D., T. E. Eling, Ph.D., M. W. Anderson, Ph.D. (Environmental Biometry Branch), S. Vicinus, B. S.

COOPERATING UNITS: Environmental Biometry Branch

MAN YEARS

Total: 0.8
Professional: 0.5
Other: 0.3

PROJECT DESCRIPTION

OBJECTIVES: (1) To study uptake and disposition of dieldrin by the isolated perfused rabbit lung preparations, (2) to investigate the initial velocity of uptake of aldrin and dieldrin by the lung, (3) to determine if any significant residual amount of either aldrin or dieldrin was retained by the lung, and (4) the extent and rate of binding in accumulation of these chemicals by the lung.

METHODS EMPLOYED: Rabbit lungs were surgically removed and maintained in a recirculating apparatus. An artificial medium was used as perfusate. Dieldrin was introduced in 50 μ l of ethanol after the lung had equilibrated for 15 minutes. Samples of the perfusate were taken at predetermined time points. Experiments were terminated after 60 minutes and the lung homogenized. Samples of the lung homogenate and perfusate were extracted and analyzed for dieldrin and other possible metabolites -- using gas chromatography.

For another series of experiments designed to determine initial velocity of uptake of aldrin and dieldrin by the lung, the perfusate effluxing

out of the lung was collected as a series of timed samples in toto. Predetermined quantity of the desired chemical was mixed with the perfusate using a servo-control infusion pump and at the end of the infusion period fresh perfusate free of the chemical was used to determine the efflux rate for the chemical from the lung.

MAJOR FINDINGS: Dieldrin was rapidly taken up by the lung from the recirculating perfusate. A steady-state condition was established following this rapid uptake, and the dieldrin concentration in the perfusate remained constant at times beyond 6 minutes after the addition of dieldrin to the perfusate. Pulmonary accumulation of dieldrin was dependent on its concentration in the perfusate. This was determined to be a biphasic uptake: a saturable component of uptake giving way to a non-saturable component at higher concentrations of dieldrin in the perfusate. The uptake of aldrin into and efflux of aldrin and dieldrin from the isolated perfused lung were examined over a range of aldrin concentrations (Cp). The rate of aldrin uptake decayed exponentially in the early part of the experiment and then reached a constant value toward the end of the influx period. These data were fitted to a curve with one component and a constant (M). Where M was plotted against Cp, M reached a plateau indicating a saturable process. This process was probably the metabolism of aldrin to dieldrin by the lung. Aldrin effluxed from the lung mono-exponentially when perfusate free of the chemical was perfused through the lung at the end of aldrin influx. The rate of dieldrin uptake by the lung exhibited a biexponential decay. Initial velocity of both of these uptake components, A + B, were independent of the perfusate concentration of dieldrin. The amount of dieldrin accumulated by perfused lung preparations varied with the perfusate concentration of dieldrin (Cp) and the influx time. The fast decay component of dieldrin uptake vs. the slow decay component of dieldrin uptake represented the non-saturable vs. saturable components of uptake by the lung, respectively. The efflux rate data for dieldrin could be resolved into two exponential functions, indicating that dieldrin in the lung was released from two distinct pools. The pool matches from the influx and the extrapolated efflux data indicated that no significant amount of dieldrin residues were likely to remain in the lung when the perfusate concentrations of dieldrin fell to zero.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: There is considerable interest concerning interaction of epoxides with various physiological systems. This pharmacokinetic study was carried out with aldrin and dieldrin in order to understand the nature and the mechanism of interaction of lung with these cyclodiene pesticides. The major findings of this investigation indicate that although aldrin is rapidly converted to its epoxide dieldrin by the lung, and both aldrin and dieldrin are taken up by the lung from the circulating blood, neither aldrin nor dieldrin are likely to remain in the lung to any significant extent once pesticide exposure ceases.

PROPOSED COURSE: The project was terminated.

KEYWORD DESCRIPTORS

Aldrin; dieldrin; isolated perfused rabbit lung; xenobiotic metabolism and storage; pharmacokinetics of uptake and release; lung-epoxide interactions; lung storage of chemicals.

PUBLICATIONS

Mehendale, H. M., El-Bassiouni, E. A., and McKinney, J. D.: Disposition of aldrin by isolated perfused rabbit lung preparations. FASEB, Vol. 33, Issue 3, p. 534. (Abstract)

Project No.: Z01 ES 80014-02 PB

1. Pharmacology Branch
- 2.
3. Research Triangle Park, N. C.

PHS-NIH

Individual Project Report

July 1, 1974 through June 30, 1975

PROJECT TITLE: "Metabolism of Polychlorinated Biphenyls"

PREVIOUS SERIAL NUMBER: NIEHS-P-016

PRINCIPAL INVESTIGATOR: H. B. Matthews, Ph.D.

OTHER INVESTIGATORS: L. T. Jao, Ph.D., M. Clemons

COOPERATING UNITS: None

MAN YEARS:

Total:	1.4
Professional:	1.3
Other:	0.1

PROJECT DESCRIPTION

OBJECTIVES: To determine the importance of position and degree of chlorination of the PCB's to their rate of metabolism and the nature of the metabolites formed.

METHODS EMPLOYED: The studies have utilized ^{14}C -labeled PCB isomers and their in vivo metabolism by the rat to obtain the necessary metabolites. The metabolites were isolated by various extraction techniques, purified by thin-layer and column chromatography and identified by mass spectral and nuclear magnetic resonance analysis.

MAJOR FINDINGS: (1) The major metabolites of the PCB's are mono- and dihydroxylated PCB's and there is a possibility of an epoxide intermediate. (2) Metabolism appears to be a prerequisite to PCB excretion. (3) Two adjacent unsubstituted carbon atoms are necessary for an appreciable rate of metabolism of the PCB's.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The polychlorinated biphenyls are ubiquitous environmental contaminants, and knowledge of their biotransformation and the identification of their metabolites is essential to our understanding of their importance as potential hazards to man and his environment. We have evidence that

epoxides are formed in the metabolism of PCB's. Epoxides have been implicated in a number of undesirable biological reactions including teratogenesis, carcinogenesis and tissue necrosis.

PROPOSED COURSE: Continue the study of the metabolism of additional PCB isomers in an effort to determine the importance of position and degree of chlorination on the rate and nature of metabolite formation.

KEYWORD DESCRIPTORS

Polychlorinated biphenyls (PCB's); PCB metabolism in vivo; excretion of PCB's; epoxides.

Project No.: Z01 ES 80015-02 PB

1. Pharmacology Branch
- 2.
3. Research Triangle Park, N. C.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

PROJECT TITLE: "Transport of Xenobiotics by Blood and Its Constituents"

PREVIOUS SERIAL NUMBER: NIEHS-P-015

PRINCIPAL INVESTIGATOR: H. B. Matthews, Ph.D.

OTHER INVESTIGATORS: M. W. Anderson, Ph.D., M. Fields; J. Younger

COOPERATING UNIT: Environmental Biometry Branch

MAN YEARS

Total:	0.3
Professional:	0.1
Other:	0.2

PROJECT DESCRIPTION

OBJECTIVES: The blood is responsible for the transport of many endogenous substances throughout the body. It also carries either free or bound to its constituents most of the xenobiotics to which the organism is exposed. These studies were designed to first determine the mechanism of xenobiotic transport and secondly to determine if this transport in any way interferes with the normal transport of endogenous materials.

METHODS EMPLOYED: The distribution of ¹⁴C-labeled xenobiotics such as biphenyl, four different polychlorinated biphenyls (PCB's), three chlorinated pesticides, one unchlorinated pesticide and an endogenous compound, a steroid hormone are being studied in various blood fractions. Methods employed utilized adsorption and molecular sieve chromatography, density gradient centrifugation and electrophoresis.

MAJOR FINDINGS: (1) Initially a large portion (up to 60%) of an iv dose of the PCB's was associated with the red blood cells. (2) The remaining 40% of an iv dose of the chlorinated compounds was distributed between the albumin and lipoproteins. (3) Red blood cells released their associated PCB's most rapidly followed by the lipoproteins. (4) The percent of the total dose of a xenobiotic found to be associated with the blood lipoproteins is a function of its chlorination (or lipid solubility).

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE:

Environmental exposure to a large number of xenobiotics via air, water or food contamination is significant. These materials are transported by the blood to various detoxication, excretion or storage sites within the body. Thus, if the transport of any one of these materials should interfere with the normal transport of endogenous materials by the blood then it could result in an imbalance of homeostasis and serve as a mechanism of toxic effect.

PROPOSED COURSE: Current plans call for similar studies of PCB metabolites and several more non-chlorinated compounds, and studies of the effect of chlorinated and non-chlorinated exogenous compounds on the blood transport of endogenous compounds, e.g., certain hormones.

KEYWORD DESCRIPTORS

Red blood cells; plasma proteins (albumin, lipoproteins); blood proteins-xenobiotic interactions; xenobiotic; polychlorinated biphenyl (PCB).

Project No.: Z01 ES 80016-02 PB

1. Pharmacology Branch
- 2.
3. Research Triangle Park, N. C.

PHS-NIH

Individual Project Report

July 1, 1974 through June 30, 1975

PROJECT TITLE: "Pharmacokinetics of Chlorinated Xenobiotics"

PREVIOUS SERIAL NUMBER: NIEHS-P-014

PRINCIPAL INVESTIGATOR: H. B. Matthews, Ph.D.

OTHER INVESTIGATORS: M. W. Anderson, Ph.D. (Environmental Biometry Branch),
M. Fields, J. Younger

COOPERATING UNIT: Environmental Biometry Branch

MAN YEARS

Total:	2.4
Professional:	0.9
Other:	1.5

PROJECT DESCRIPTION

OBJECTIVES: To develop a pharmacokinetic model which will predict, for a number of species, the distribution, accumulation and excretion of polychlorinated biphenyls (PCB's) and other chlorinated xenobiotics after different dose levels and routes of exposure.

METHODS EMPLOYED: The distribution, accumulation, and excretion of four ¹⁴C-labeled PCB's (4-chloro-, 4,4'-dichloro-, 2,4,5,2',5'-penta-chloro- and 2,4,5,2',4',5'-hexachlorobiphenyl) were studied after iv and oral administration to rats. The major tissues and excreta were sampled at times varying from a few minutes to several weeks post-treatment. Distribution and excretion were studied under conditions of normal feeding and starvation. Analyses were facilitated by the use of a biological material oxidizer and liquid-scintillation counting. All of the data were subjected to further analysis by computer.

MAJOR FINDINGS: (1) Each of the PCB's was rapidly removed from blood. (2) Initial storage was primarily in the liver and muscle. (3) Long-term storage was in the skin and adipose tissue. (4) Storage of metabolites in skin and adipose tissue accounted for a negligible portion of the total PCB dose. (5) Where two adjacent carbon atoms were present, the

excretion of the PCB's was inversely proportional to their degree of chlorination.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE:

Chlorinated xenobiotics are among the most persistent of our environmental contaminants. These compounds are known to cause a variety of biological disorders in man as well as being at least partially responsible for the declining numbers of certain species of wildlife. Accumulation of high concentrations in the tissues of animals exposed to relatively low doses is a characteristic of certain chlorinated xenobiotics. Yet many of these compounds are used in our current methods of food production, disease control and certain industrial processes. Thus, we need to be able to predict what will be the disposition of these compounds in animals and man. Understanding the pharmacokinetics of PCB's in different species will help in this prediction or extrapolation of animal data to man.

PROPOSED COURSE: We plan to study the distribution, accumulation and excretion of at least one additional PCB in the rat in order to confirm the findings listed above. Then we plan to study the distribution and excretion of several of these compounds in at least two other species and to utilize the data obtained to construct a mathematical model for the disposition of PCB's which we hope will allow the extrapolation of the data obtained for the PCB's in a limited number of species to other species and other classes of chlorinated xenobiotics.

KEYWORD DESCRIPTORS

Polychlorinated biphenyl (PCB); tissue distribution of PCB; storage and accumulation of PCB; excretion of PCB; pharmacokinetic models; inter-species extrapolation of PCB disposition; starvation; liver; muscle; skin; fat.

PUBLICATIONS

Matthews, H. B. and Anderson, M. W.: The effect of chlorination on the distribution and excretion of polychlorinated biphenyls. Society of Toxicology Meeting, Williamsburg, Va., 1975. (Abstract)

Matthews, H. B. and Anderson, M. W.: Distribution and kinetics of 2,4,5, 2',5'-pentachlorobiphenyl in the male rat. Drug Metab. Disp. to be published in May-June issue, 1975.

Matthews, H. B., Chen, P. R., Mehendale, H. M., and Anderson, M. W.: The metabolism, storage, and excretion of highly chlorinated compounds by mammals. In Kohn, G. K. (Ed.): ACS Symposium Series #2, American Chemical Society, 1974, pp. 54-68.

Project No.: Z01 ES 80017-04 PB

1. Pharmacology Branch
- 2.
3. Research Triangle Park, N. C.

PHS-NIH

Individual Project Report

July 1, 1974 through June 30, 1975

PROJECT TITLE: "The Inhalation Toxicity of Compounds Common to Household Aerosols"

PREVIOUS SERIAL NUMBER: NIEHS-P-018

PRINCIPAL INVESTIGATOR: R. T. Drew, Ph.D.

OTHER INVESTIGATORS: B. N. Gupta, Ph.D. (Environmental Biology and Chemistry Branch), Galen Hasler, M.D., George J. Taylor, M.D., John O'Neil, Ph.D., M. T. Riley, J. Schroeder, F. Harrington, W. Gibson, R. Walden

COOPERATING UNITS: Environmental Biology and Chemistry Branch (Pathology and Histology), Environmental Biometry Branch

MAN YEARS

Total:	3.3
Professional:	1.2
Other:	2.1

PROJECT DESCRIPTION

OBJECTIVES: To determine acute and chronic inhalation toxicity of selected compounds common to household aerosols and to identify any potential synergistic interactions. Compounds previously investigated include Rehydrol ASC (a propylene glycol complex of aluminum-chloride-hydroxide used in aerosol deodorants), "Airwick," hexachlorophene, dichlorodifluoromethane (F-12) a commercial preparation of hair spray, and a commercial spray adhesive. Compounds presently under investigation include Micro-dry (another aluminum-chloride-hydroxide compound not complexed with propylene glycol), several commercial preparations of aerosol oven cleaners, F-12, and trichlorofluoromethane (F-11).

METHODS EMPLOYED: 1. Exposure techniques: Exposures are carried out under dynamic conditions with contaminant concentrations being monitored frequently during exposures. Aerosols of aluminum-chloride-hydroxide are generated from an aqueous solution-with a compressed air-driven nebulizer. Commercial aerosols are ejected into an elutriation chamber

which traps the larger particles and then into an exposure chamber. The cans are activated mechanically on a 5-second on - 25-second off cycle. The fluorocarbons are blended with compressed air to the concentration desired and then directed to the chamber or in some cases to an endotracheal tube for direct exposure.

2. Toxicity evaluation: Several techniques are utilized to determine the effects of exposures. These include histologic preparation of tissue and microscopic examination for pathological or morphological changes; investigation of mixed-function oxidase (MFO) activities of both lung and liver microsomes after animal exposure; assessment of weight gain and organ weights after exposure; investigation of any change in respiratory physiological parameters such as breathing rate and volume, resistance, and static and dynamic compliance; measurement of electrocardiographic and hemodynamic parameters, and myocardial contractility changes after and during exposures; and measurement of deposition and clearance of chemicals in the lung, and body distribution and elimination of chemicals after exposure.

MAJOR FINDINGS: 1. Animal exposure to either compound of aluminum for 3 days causes increases in lung weights in hamsters, rats and rabbits. Exposure of rats and hamsters to aluminum-chloride-hydroxide at a concentration of 50 mg/m³ for 6 hrs/day, 5 days/week for 30 days induces permanent changes. In rats a year later, the lungs of exposed animals are obviously larger and weigh about twice as much as lungs from air-exposed control animals. Histologically, lesions are present which may precede emphysema. The half-life of aluminum in the lungs is about 30 days in hamsters and 60 days in rats.

2. Oven Cleaners. Of 5 brands initially investigated, 3 were chosen for study. Rats and hamsters were exposed to 2 cans per day for 1, 2, 3 or 4 days and sacrificed 14 days later. Other animals were exposed to the output of 2 cans and sacrificed 1, 4, 7 or 10 days later. At sacrifice, several organs were weighed and after preparation were examined for morphological changes. No evidence of toxic effects of exposure to any of the 3 brands chosen was seen -- either in weight gain, organ-to-body weight ratios or morphological changes in lung or other organs.

3. Fluoro- and Chloro- Carbons. Acute exposures of rabbits to F-12 result in a dose related depression in left ventricular conduction and cardiac output without changes in heart rate, blood pressure, arterial PO₂, PCO₂ or pH. Intermittant exposure to 10% F-12 does not alter the response to an acute challenge of F-12 suggesting effects are not cumulative. In a study comparing cardiac depression by F-11, F-12, halothane, dichloromethane, chloroform and 1,1,1-trichloroethane, F-12 and dichloromethane had less effect on cardiac output and left ventricular function than the others. As cardiac

depressants, F-11 and 1,1,1-trichloroethane appeared as potent as halothane.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The widespread use of the aerosol "spray can" dispenser has resulted in a very complex and wide variety of materials being available in respirable form. While the acute toxicity of many of the "active ingredients" in such dispensers has been investigated, little is known about the chronic toxicity of these compounds or the toxicity of combinations of these compounds and "inert ingredients" of the pressurized cans. Elucidation of the chronic toxicity of these compounds and their potential combinations can be of vital importance to environmental health.

PROPOSED COURSE: The studies on aluminum are being completed. Studies on fluorocarbons will continue, with continued use of the cardiomyopathic hamster, investigating whether the increased susceptibility to F-11 is unique to F-11 or common to all fluorocarbons. Other commercial products may be investigated.

KEYWORD DESCRIPTORS

Household aerosol toxicity; aluminum-chloride-hydroxide; aerosol oven cleaner toxicity; aerosol antiperspirant toxicity; fluorocarbons; lung histopathology; enzymology; cardiovascular effects of fluorocarbons; respiratory physiology and toxicology; dichloromethane; halothane; chloroform.

PUBLICATIONS

Drew, R. T.: Testified before FDA Over-the-Counter panel on anti-perspirants.

Drew, R. T.: Inhalation studies on compounds common to household aerosols. Presented to Inter-Industry Aerosol Safety Conference. Cosmetic, Toiletry, and Fragrance Association, Washington, D.C. 1974.

Drew, R. T., Gupta, B. N., and Riley, M. T.: Inhalation studies with aerosol oven cleaners. To be presented at the American Industrial Hygiene Association, 1975. (Abstract)

Drew, R. T. and Taylor, G. J.: Cardiophysiological studies with stressed animals. Proceedings of the 5th Annual Conference on Environmental Toxicology held in Dayton, Ohio, September 24-26, 1974 (in press).

Taylor, G. J.: Cardiac arrhythmias in hypoxic rabbits during aerosol propellant inhalation. Arch. Environ. Hlth. In press.

Taylor, G. J. and Drew, R. T.: Cardiomyopathy predisposes hamsters to aerosol propellant toxicity. Toxicol. Appl. Pharmacol. In press.

Taylor, G. J. and Drew, R. T.: Cardiovascular effects of chronic and acute fluorocarbon 12 exposures on rabbits. J. Pharm. Exptl. Ther. 192: 129-135, 1975.

Project No.: Z01 ES 80018-05 PB

1. Pharmacology Branch
- 2.
3. Research Triangle Park, N. C.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

PROJECT TITLE: "The Effects of Drugs and Chemicals on the Inhalation Toxicity of Chemical Solvents"

PREVIOUS SERIAL NUMBER: NIEHS-PT-019

PRINCIPAL INVESTIGATORS: R. T. Drew, Ph.D. and C. Harper, Ph.D.

OTHER INVESTIGATORS: J. R. Fouts, Ph.D., J. Patel, Ph.D., M. Hogan, Ph.D.,
B. Gupta, Ph.D., F. Talley, M. T. Riley, J. Schroeder,
W. Gibson, F. Harrington, R. Walden, and V. Hyatt

COOPERATING UNITS: Environmental Biology and Chemistry Branch (Histology),
Environmental Biometry Branch

MAN YEARS

Total:	5.0
Professional:	2.2
Other:	2.8

PROJECT DESCRIPTION

OBJECTIVES: Liver is known to contain microsomal toxication and detoxication enzymes that are inducible by certain drugs and other chemicals. There are suggestions that analogous lung enzymes are also inducible. Detoxication of some organic solvents is catalyzed by some of these microsomal enzymes. This study has the following objectives:

1. Determine if pretreatment of animals with drugs and other chemicals which modify liver and/or lung microsomal enzymes will alter the acute and chronic inhalation toxicity of selected organic chemicals and solvents including benzene, xylene, and vinyl chloride.
2. Determine whether pretreatment (by inhalation) by common solvents affects their enzymatic degradation in lung and liver.
3. Identify the various metabolic pathways involved in the enzymatic degradation of paraxylene and determine whether pretreatment by known inducers stimulates different metabolic pathways.

4. Identify urinary metabolites for use as biological indicators of paraxylene exposure.

5. To investigate the fate of benzene in in vitro systems when excess product (phenol) is added to microsomal preparations from pulmonary or hepatic tissue.

METHODS EMPLOYED: Inhalation exposures are carried out in chambers specifically designed for such studies. They are operated under dynamic conditions of air flow and atmospheric concentrations of specific compounds are monitored at frequent intervals. Biochemical assays are carried out in routine fashion.

MAJOR FINDINGS: 1. Reliable reproducible methods have been developed to assay for both benzene and paraxylene metabolism by microsomes prepared from lungs and livers of several species. Comparative studies on rates of metabolism of these two substrates by microsomes prepared from both liver and lungs from rats, rabbits, and hamsters have been completed.

2. Animal pretreatment by known hepatic microsomal enzyme inducers increased metabolism of benzene by liver microsomes from both the rat and the rabbit while not affecting metabolism of benzene by lung microsomes. These enzyme inducers did not affect the acute toxicity of inhaled or injected benzene in rats.

3. Chronic exposure of rats to benzene vapors (1,650 ppm, 6 hrs/day 5 days/week for 12 weeks) produced a depressed growth rate, a diminished lymphocyte count and a slight increase in the in vitro rate of hepatic microsomal metabolism of benzene. Continuous administration of phenobarbital in the drinking water of rats given the same benzene inhalation exposure regime partially reversed the diminished growth rate, partially protected against the lymphocytopenia and markedly increased the rate of hepatic microsomal metabolism of benzene.

4. Phenol was a competitive inhibitor of benzene hydroxylation in both pulmonary and hepatic microsomal preparations.

5. Three 4-hour exposures of female rats to 4000 ppm benzene doubled the benzene hydroxylase activity in hepatic microsomal preparations with no apparent increase in cytochrome P-450. Benzene hydroxylase activity in pulmonary microsomal preparations was not changed after these exposures.

6. Urinary metabolites of p-xylene from female rats included both p-toluic acid and 2,5-dimethyl phenol.

7. Hepatic and pulmonary microsomal preparations of female rats metabolized p-xylene exclusively to p-methylbenzyl alcohol. This alcohol is converted to p-toluic acid by soluble enzymes from hepatic fractions but not from pulmonary fractions. These soluble enzymes may be replaced, at least partially, by commercially available alcohol dehydrogenase and aldehyde dehydrogenase.

8. Female rats, when treated with p-xylene, showed no change in p-xylene metabolism by hepatic microsomes but a 60% loss in p-xylene metabolizing activity by pulmonary microsomes. The loss in pulmonary xylene metabolizing enzyme activity was observed with three routes of xylene administration (IP, oral and inhalation). Maximum loss of xylene metabolizing enzyme activity was observed two hours after xylene treatment was discontinued. Recovery to normal activity was observed eight days after treatment was discontinued. Phenobarbital increased p-xylene metabolism by hepatic microsomes but did not affect enzyme activity in pulmonary microsomes. Treatment with phenobarbital or antioxidants (vitamin E) did not modify the loss in activity by pulmonary microsomes from xylene-exposed rats.

9. Male rabbits exposed to 1000 ppm p-xylene for 4 hours showed a complete loss of CO-binding pigment (cytochrome P-450) in pulmonary microsomes, but not in hepatic microsomes. This change in P-450 was accompanied by a loss of p-xylene metabolizing activity by rabbit pulmonary microsomes but not rabbit hepatic microsomes.

10. Rats pretreated with phenobarbital and exposed for up to ten days to 13,500 ppm vinyl chloride showed marked weight loss after the second exposure. Occasional morphological changes were also seen in the livers of these animals. Phenobarbital alone or vinyl chloride alone had no effect on liver histology or body weight.

11. The carboxyhemoglobin/oxyhemoglobin (CO-Hb/Ox-Hb) ratios in blood have been characterized in rabbits as a function of duration and concentration of dichloromethane (DCM) exposure. After a single 20-minute exposure to DCM vapors, the ratio CO-Hb/Ox-Hb increases for the first 2-3 hours to a maximum and gradually declines to basal levels 7-8 hours after the exposure. Animal treatment with either phenobarbital, an MFO inducer, or carbon tetrachloride, an MFO inhibitor, lowered the ratio, CO-Hb/Ox-Hb, in DCM-exposed animals as compared with DCM exposure alone. Animal treatment with several other inducers and inhibitors of hepatic mixed-function oxidases did not alter the course of the DCM-induced change in blood CO-Hb/Ox-Hb ratios.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: In today's society the number of people taking drugs either prescribed by a physician or illicitly is substantial. Some of these compounds stimulate liver microsomal enzymes, and possibly lung systems, thus increasing the rate at which certain chemicals and/or other endogenous

substrates are metabolized. These studies are designed to better define the toxic responses to selected solvents and to determine if the toxicity of these solvents can be altered by pre-exposure to themselves or to other drugs. The effect of the route of administration is also being studied.

Benzene has been chosen for study because of its widespread industrial and household use and because it is now being proposed for use instead of Tetraethyl lead in increasing gasoline octane ratings. Paraxylene was chosen because it is also high on the list of widely-used solvents and because little information is known about its toxicity. Vinyl chloride has been investigated because of the recent finding that this compound is a carcinogen. The recent discovery that dichloromethane (a widely-used solvent and degreaser) was metabolized directly to carbon monoxide by the liver prompted studies with this compound.

PROPOSED COURSE: Solvent toxicity and metabolism and the modification thereof by known inducers and inhibitors of chemical metabolism in liver and other tissue will continue to be investigated. Particular emphasis will be placed on identification of other metabolites from paraxylene and on the pathways of metabolism stimulated by certain inducers. An attempt will be made to identify urinary metabolites. Studies will begin with vinyl bromide, measuring effects of exposure to vinyl bromide in normal and phenobarbital-treated rats. Attempts will also be made to alter the relative activities of toxifying (MFO) vs. detoxifying (epoxide hydrase, GSH-conjugation) pathways for these solvents in different tissues so as to assess the role of metabolism in solvent-induced toxicity and specific tissue effects of solvents and chemicals that are inhaled.

KEYWORD DESCRIPTORS

Solvent toxicity; benzene; p-xylene; dichloromethane; vinyl chloride; microsomal enzyme induction and inhibition; solvent metabolism; carbon monoxide; phenol; vitamin E; pulmonary and hepatic chemical metabolism; inhalation toxicity; carboxyhemoglobin; CCl_4 ; phenobarbital.

PUBLICATIONS

Drew, R. T., Harper, C., Gupta, B. N., and Talley, F. A.: The effects of vinyl chloride exposure of rats pretreated with phenobarbital. Environ. Hlth. Persp. in press.

Harper, C: p-Xylene metabolism by rat pulmonary and hepatic microsomes. Fed. Proc. 34, 1975. (Abstract)

Harper, C., Drew, R. T., and Fouts, J. R.: Inhalation of benzene: toxicity and metabolism. Proceedings of International Symposium on Active Intermediates: Formation, Toxicity, and Inactivation. Turku, Finland, July, 1975. (Abstract)

Roth, R. P., Drew, R. T., Lo, R. J., and Fouts, J. R.: Dichloro-
methane inhalation, carboxyhemoglobin levels and drug metabolizing
enzymes in rabbits. Toxicol. Appl. Pharmacol. in press.

Project No.: Z01 ES 80019-01 PB

1. Pharmacology Branch

2.

3. Research Triangle Park, N. C.

PHS-NIH

Individual Project Report

July 1, 1974 through June 30, 1975

PROJECT TITLE: "Intermediary Metabolism of Pulmonary Tissue Studied In Vitro"

PREVIOUS SERIAL NUMBER: None

PRINCIPAL INVESTIGATOR: J. J. O'Neil, Ph.D.

OTHER INVESTIGATOR: Frank Harrington

COOPERATING UNIT: None

MAN YEARS

Total: 0.5

Professional: 0.4

Other: 0.1

PROJECT DESCRIPTION

OBJECTIVES: Intermediary metabolism, although generally well characterized for most tissues, has not been thoroughly studied for the lung. This study will define substrate utilization by lung tissue, that is, the uptake, oxidation, and incorporation of glucose, amino acids, and fatty acids.

METHODS EMPLOYED: Substrate utilization studies will be performed with lung tissue slices.

MAJOR FINDINGS: Special techniques for using lung tissue slices have been developed. Techniques in tissue handling, slice thickness, shaking rate, gas phase, and method of weight estimation have been varied to determine optimal conditions for estimating metabolism while maintaining a normal physiological milieu.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE:

Although the lung is the first and primary organ exposed to airborne toxicants, surprisingly little is known regarding pulmonary intermediary metabolism and changes which might occur in response to insults. It is apparent, that in addition to gas exchange, the lung plays a vital role in the removal, metabolism and release of substances in the blood as

well as the synthesis and secretion of substances peculiar to the lung. An understanding of the intermediary metabolism of pulmonary tissue may contribute to our understanding of normal lung function and to changes which occur early in response to toxicant exposure or disease.

PROPOSED COURSE: (1) Evaluate the relative contribution of various substrates (e.g., glucose, amino acids, free fatty acids) to meet the energy requirements of lung tissue, (2) estimate the relative utilization or requirement for various substrates in the presence or absence of other substrates (e.g., difference in glucose utilization in the presence or absence of amino acids), and (3) estimate changes in metabolism produced by metabolic inhibitors and by toxic substances inhaled or administered to animals.

KEYWORD DESCRIPTORS

Amino acid utilization; fatty acid utilization; glucose utilization; intermediary metabolism; pulmonary metabolism; substrate utilization; tissue slices; pollutant effects on lung metabolism.

Project No.: Z01 ES 80020-03 PB

1. Pharmacology Branch
- 2.
3. Research Triangle Park, N. C.

PHS-NIH

Individual Project Report

July 1, 1974 through June 30, 1975

PROJECT TITLE: "Changes in Mammalian Pulmonary Function Produced by Inhaled Environmental Agents"

PREVIOUS SERIAL NUMBER: NIEHS-P-020

PRINCIPAL INVESTIGATORS: J. J. O'Neil, Ph.D and R. T. Drew, Ph.D.

OTHER INVESTIGATORS: None

COOPERATING UNIT: None

MAN YEARS:

Total: 0.4

Professional: 0.4

Other: 0.0

PROJECT DESCRIPTION

OBJECTIVES: Techniques are being developed to measure changes in the pulmonary function of small mammals after inhalation exposure to oxidant gases and pulmonary irritants. These changes are being correlated with data from cardiovascular and histological investigations.

METHODS EMPLOYED: A whole body pressure plethysmograph has been constructed and is being used to estimate the pulmonary volumes of small laboratory animals. From these data it is possible to calculate pulmonary resistance and compliance which are indicators of pulmonary airway or parenchymal changes.

MAJOR FINDINGS: Plethysmographic equipment has been developed and calibrated and typical *in vivo* values for pulmonary compliance and resistance have been estimated. These data provide a basis for evaluation of pulmonary changes when small animals are exposed to inhaled toxicants.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Changes in pulmonary function of animals after exposure to inhaled environmental agents are known, in many cases, to be associated with

cellular damage and morphologic changes. Development of sensitive methods to detect pulmonary function changes may provide an early marker of lung damage and provide insight into both the mechanism of pulmonary damage and the types or location of the cells most readily injured by specific agents.

PROPOSED COURSE: Future research will focus on three main areas:

(1) In order to establish what might be considered normal *in vivo* values, lung function data will be derived from significant quantities of unexposed animals of various ages. (2) These data will be compared to those from animals in whom functional changes may have occurred either after chronic or during acute exposures to pulmonary irritants. (3) Changes in pulmonary function will be related to any changes in lung morphology or in lung vascular or cardiac functions.

KEYWORD DESCRIPTORS

Inhalation exposure; oxidants (gases); pressure plethysmograph; pulmonary compliance; pulmonary function tests; pulmonary irritants; pulmonary resistance; respiratory physiology; cardiovascular functions (lung, heart).

Project No.: Z01 ES 80021-01 PB

1. Pharmacology Branch

2.

3. Research Triangle Park, N. C.

PHS-NIH

Individual Project Report

July 1, 1974 through June 30, 1975

PROJECT TITLE: "Studies of Membrane Glycoproteins in Normal and Neoplastic Tissues"

PREVIOUS SERIAL NUMBER: None

PRINCIPAL INVESTIGATOR: R. P. DiAugustine, Ph.D. and P. E. Voytek, Ph.D.

OTHER INVESTIGATORS: Dolores Eckberg, B. S. and T. Abe, Ph.D.

COOPERATING UNIT: None

MAN YEARS

Total: 0.4

Professional: 0.2

Other: 0.2

PROJECT DESCRIPTION

OBJECTIVES: Glycoproteins of cell surfaces apparently have an important role in antigenicity, cell growth, and cytodifferentiation and cellular recognition. The objectives of the present study are to elucidate the biochemical, immunochemical, and structural properties of intact membrane glycoproteins, especially those of the plasma membrane, from both normal and neoplastic cells. Presently, we are studying the enzyme nicotinamide adenine dinucleotide glycohydrolase (E.C. 3,2,2.5.) which has a high specific activity in the plasma membrane fraction obtained from rat liver homogenates.

METHODS EMPLOYED: Ultracentrifugation, column chromatography, isoelectric focusing, SDS and polyacrylamide gel electrophoresis, amino acid analysis, and gas-liquid chromatography.

MAJOR FINDINGS: NAD glycohydrolase was solubilized from a preparation containing rat liver microsomal and plasma membranes as judged by suitable enzyme markers. Solubilization was achieved by a combination of sonication, freeze-thawing, and digestion with crude pancreatic lipase. Although the lipase preparation was essential for solubilization, it appears that the lipase per se is not the solubilizing enzyme in the

crude pancreatic preparation. Approximately 80% of the membrane-bound enzyme was solubilized. The enzyme in the supernatant of the digest was then purified by DEAE cellulose and Sephadex G-150 columns and then by isoelectric focusing using a pH 6-8 ampholine. Multiple peaks were obtained on focusing which do not appear to be an artifact of preparation. A major peak occurred at a pI of 7.8. Using this peak, we obtained a 1000-fold purification of the enzyme. This protein was found to have a m.w. of about 83,000 daltons, as measured using sucrose gradient centrifugation. The pI 7.8 enzyme protein was stained with periodic acid-Schiff reagent on electrophoresis gels. The protein contained about 10% hexoses (of the total dry weight). Enzyme activity was slightly inhibited by concanavalin A but not by wheat germ agglutinin or fucose-binding protein.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: There is much evidence to suggest that aberrations at the surface of cells might be responsible for loss of controlled growth or "contact inhibition." These aberrations could occur as modified, blocked, or deleted functional groups of proteins or intact macromolecules. Changes in the specific sugar sequence or "sugar code" could introduce aberrations in proteins. Membranes, as a result of a point mutation of a single protein, can be affected drastically by a cascade of events because they are highly ordered and condensed organelles. The present studies have been undertaken to give basic knowledge about a class of proteins in membranes of normal and tumor cells; these proteins have been strongly implicated as having regulatory roles in metabolism and growth. We believe that accurate observations and clarification of the aberrations of tumor cells rests with the understanding of the biochemistry and structure of intact membrane molecules.

PROPOSED COURSE: (1) To complete studies of the amino acid and carbohydrate compositions of NAD glycohydrolase, (2) to examine the re-association of the enzyme glycoprotein with plasma membranes. What role do the carbohydrate residues have in association of the enzyme with membranes? (3) to study the isoelectric focusing profile of the enzyme from purified plasma membranes vs. microsomes plus plasma membranes, (4) to make the antibody to the enzyme glycoprotein and examine the immunochemical properties of the protein in normal liver and several hepatomas, and (5) to continue to develop methods for the solubilization and isolation of other membrane glycoproteins.

KEYWORD DESCRIPTORS

Glycoproteins; NAD glycohydrolase; plasma membrane; tumor; liver; microsomes.

1. Pharmacology Branch
- 2.
3. Research Triangle Park, N. C.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

PROJECT TITLE: "Hepatic Transglutaminase"

PREVIOUS SERIAL NUMBER: NIEHS-P-025

PRINCIPAL INVESTIGATORS: R. P. DiAugustine, Ph.D. and T. Abe, Ph.D.

OTHER INVESTIGATOR: Dolores Eckberg, B. S.

COOPERATING UNIT: None

MAN YEARS

Total:	1.5
Professional:	1.2
Other:	0.3

PROJECT DESCRIPTION

OBJECTIVES: Transglutaminase is an enzyme of unknown function in the liver. This enzyme, however, can couple (covalently) in vitro selected amines such as phenethylamine, isoniazid, and putrescine to certain endogenous proteins. Calcium is a requisite for this reaction. This enzyme activity appears operative in plasma as Factor XIII for the catalysis of the $\epsilon(\gamma\text{-glutamyl})$ lysine bonds between fibrin monomers. Factor XIII prior to the sequential clotting reactions, exists -- unlike the liver enzyme -- as a zymogen in plasma. There is some evidence to indicate that the hepatic enzyme might function in vivo in the manner of Factor XIII by catalyzing $\epsilon(\gamma\text{-glutamyl})$ lysine bonds for membrane proteins (P. J. Birkbichler et al. Biochim. Biophys. Acta 291: 149, 1973).

The objectives of the present program are to: (1) purify and to characterize rabbit liver transglutaminase, (2) determine what proteins in the cytosol and membrane fractions of the hepatocyte can act as acceptors for amines such as putrescine, spermine, isoniazid and tyramine, (3) determine any role of Ca^{++} or other divalent metals in the stimulation of hepatic intracellular transglutaminase. Unlike the blood enzyme (Factor XIII), hepatic transglutaminase needs no proteolysis to initiate activity. However, it is difficult to explain how transglutaminase is operative in vivo when intracellular calcium is so very low in concentration ($\sim 1 \times 10^{-6}$ M), and (4) to identify any intracellular proteins which are cross-linked by hepatic transglutaminase and to examine any structural

or dynamic roles of these proteins.

METHODS EMPLOYED: Column chromatography, isoelectric focusing, polyacrylamide gel electrophoresis, ultracentrifugation, paper chromatography, liquid scintillation spectroscopy, UV and visible spectrophotometry, amino acid analysis.

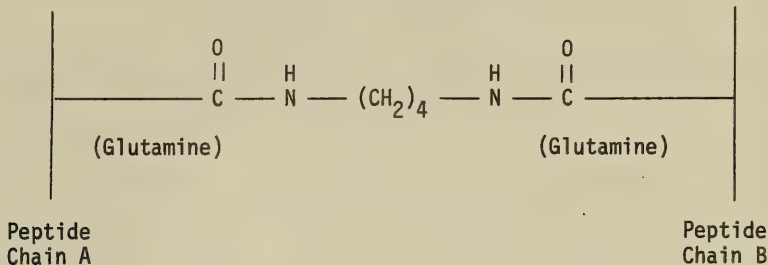
MAJOR FINDINGS: Purification: The rabbit enzyme is obtained from the cytosol of livers. The cytosol is brought to pH 5.0 with acetic acid. The precipitate is solubilized with 0.01 M potassium phosphate buffer. This solution was fractionated by $(\text{NH}_4)_2\text{SO}_4$ at 30% to 50% saturation. The fractionated sample was applied to a DEAE-Sephadex (A-50) column and eluted with 0.02 M Tris-HCl buffer, pH 7.5, using a gradient from 0.05 M KCl to 0.6 M KCl. Activity was estimated using CBZ-glutaminyglycine as acceptor and NH_2OH as the donor amine. Active enzyme fractions were combined, concentrated, and then chromatographed on a Sephadex G-200 column, followed by chromatography on DEAE-Sephadex (A-50) using a 0.2 M to 0.5 M KCl gradient. Finally, the concentrated active sample was eluted from a Sephadex G-100 column using the same Tris buffer given above.

Enzyme purity was identified as a single band by disc acrylamide gel electrophoresis and also SDS gel electrophoresis. The molecular weight of the enzyme was estimated to be 80,000 daltons by Sephadex G-200 columns and 83,000 daltons by SDS gels. Other chemical, physical and kinetic properties of the enzymes, are currently being studied.

Properties of the Endogenous Acceptor: About 3% of the total ^{14}C -putrescine substrate concentration (5 mM) is coupled to hepatic cytosol protein following incubation for 60 min at 37° in the presence of 5 mM Ca^{++} . Since 15-20 mg of cytosol protein is used in each assay, the amount of coupled amine per mg protein is estimated to be between 1 to 2 micrograms. No enzyme activity could be detected in the cytosol fraction obtained from *E. coli* or *S. epidermidis*. Incorporation of isotope in hepatic cytosol was measured using washed TCA precipitates or exhaustively-dialyzed reaction samples. Uptake of isotope was protein-dependent and followed a hyperbolic profile. Ca^{++} was obligatory for the reaction. Uptake was inhibited by spermine, isoniazid, NH_2OH , and iodoacetamide. At 5 mM iodoacetamide, no uptake of putrescine could be measured. Uptake could be enhanced by adding partially-purified transglutaminase containing no acceptor. This suggests that the putative glutamine acceptors on proteins have not been completely saturated at the plateau region of uptake. Most of the ^{14}C -putrescine radiolabeled protein acceptor is found in the void fraction following Sephadex G-150 column chromatography. Isoelectric focusing (pH 3.5-10) revealed multiple peaks between pH 4 to 7 with negligible isotope detected at $> \text{pH } 7$. A sharp maximum peak occurred at $\sim \text{pH } 3.7$. These data give properties to the cytosol acceptor as having an acid pI and

a m.w. > 200,000 daltons.

In addition to other chemical and physical properties of the acceptor proteins, we are examining the possibility that both amino terminal grouping of putrescine are utilized in the reaction. In this case, the polyamine, such as putrescine, would allow a cross-link between proteins as shown below:



SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE:

1. The fact that a number of amine-containing drugs such as isoniazid and hydralazine can be coupled to certain proteins by transglutaminase makes this enzyme a suitable candidate for drug- or chemical-induced antigen formation in vivo. Some clinical evidence has already been reported suggesting that antibodies to plasma transglutaminase are responsible for isoniazid-induced hemorrhaging.

2. Transglutaminase offers a limited method for coupling selected compounds to proteins for use in radioimmune assays.

3. Neoplastic cells apparently produce higher levels of polyamines, which can be detected in the serum. Evidence also suggests that the polyamines, especially putrescine, are coupled to cell membrane proteins which can be detected immunochemically. We suggest transglutaminase as a catalyst for such a reaction.

4. The ϵ (γ -glutamyl) lysine cross-link is a recently discovered covalent protein-protein interaction that appears to be ubiquitous in mammalian cells. If this bond can be definitely ascertained in hepatocyte plasma membranes, it would give us a new and important component to consider in the study of the physical, chemical and functional properties of membranes. What process controls the catalysis of the cross-link in membrane? Calcium influx to the cell? From where are the proteins derived to make the cross-link? What property does this cross-link give to membranes?

PROPOSED COURSE: (1) To further determine the biochemical and physical properties of rabbit liver transglutaminase, (2) to identify soluble and membrane proteins capable of acting as acceptors for transglutaminase-catalyzed reactions, (3) to identify proteins which are cross-linked by the enzyme and contain the $\epsilon(\gamma\text{-glutamyl})$ lysine, and (4) to examine Ca^{++} influx as a possible control mechanism or initiator of enzyme activity.

KEYWORD DESCRIPTORS

Calcium; transglutaminase; amines; polyamines; hepatocytes; protein cross-links; liver; chemical-induced antigen formation.

Project No.: Z01 ES 80023-03 PB

1. Pharmacology Branch
- 2.
3. Research Triangle Park, N. C.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

PROJECT TITLE: "The Composition and Origins of the Acellular Lining Layer of the Lung"

PREVIOUS SERIAL NUMBER: NIEHS-P-021

PRINCIPAL INVESTIGATORS: G. E. R. Hook, Ph.D., R. P. DiAugustine, Ph.D., and R. Mirel, M. D.

OTHER INVESTIGATORS: L. B. Gilmore, B. A. and D. L. Eckberg, B. S.

COOPERATING UNITS: Environmental Biology and Chemistry Branch (Histology and Electron Microscopy)

MAN YEARS:

Total: 1.6
Professional: 1.1
Other: 0.5

PROJECT DESCRIPTION

OBJECTIVES: Numerous chemical analyses of the acellular lung lining layer have been made and there is little doubt concerning the identity of the surfactant material obligatory for normal lung function. However, the acellular layer is also a barrier to the environment and consequently may be one of the first sites subject to attack by inhaled toxic agents. Unfortunately, apart from its lipid composition very little is known about the acellular lining layer.

The objectives of this investigation are (1) to determine the enzymatic and protein composition of the lung lining, (2) to determine the origin of these activities, and (3) to investigate compositional variations of the lining under the influence of drugs, disease and airborne pollutants.

METHODS EMPLOYED: The acellular lining is removed by lavage. The lavage is analyzed for cell, chemical and enzymic composition using microscopic, spectrophotometric, chromatographic and electrophoretic methods.

MAJOR FINDINGS: Evidence for the secretion of the lamellar organelles by the Type II cells has been primarily confined to morphological observations. Our investigations of the isolated lamellar organelles and the acellular lining layer of the lung provides the first biochemical evidence confirming that the lamellar organelles may indeed be secreted. The phospholipid composition of the lamellar organelles is similar to that found in the acellular material, consisting primarily of phosphatidyl choline, phosphatidyl glycerol and phosphatidyl ethanolamine.

Phospholipid labeling studies employing $^{32}\text{PO}_4$ indicate that phospholipids of the acellular lining could originate from the lamellar organelle and that the reverse process does not occur.

Also secreted by the Type II cell are hydrolases which are intimately associated with the secreted lamellar organelles. A number of acid and alkaline hydrolases appear to be associated with the acellular lining layer. The origins of these enzymes are currently under investigation and although many of the enzymes seem related to lysosomes, their secretion is selective. Some lysosomal enzymes are absent (β -glucuronidase, neuraminidase and fatty acid esterase) from the acellular lining layer.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The origins of the acellular lining are in doubt and our studies provide strong evidence for an exocrine function of the alveolar Type II cell.

Of considerable importance are methods which can provide evidence of disease or damage to the human lung. The origin of certain proteins or enzymes in the alveolar Type II cell might be used to assess the function of the Type II cell as well as the integrity of the vitally important acellular lining layer. For example, abnormal secretions from the Type II cell are believed to give rise to the pulmonary disease alveolar proteinosis. The biochemical abnormalities underlying diseases such as alveolar proteinosis have not been elucidated but the basic lesion may be within the enzymic composition of the lamellar organelle itself.

Diagnosis of pulmonary disease using lavage fluids could be of prime importance to human health and detection of environmental effects thereon.

PROPOSED COURSE: The properties of the protein components of the acellular lining are being investigated. The investigation of the selective secretion of enzymes to the lung lining will be extended to other species including the human if lavage samples can be obtained. Effects of pollutant exposures on composition of the acellular lining layer will be studied in animals and perhaps later in man.

KEYWORD DESCRIPTORS

Lung, acellular lining layer, composition, Type II cells, lung, hydrolases and other enzymes in lung lavage, secretion of phospholipid in lung, phospholipid biosynthesis in lung, lung lavage, lysosomes, lamellar organelles, alveolar proteinosis

PUBLICATIONS

Hook, G. E. R., Abe, T., and DiAugustine, R. P.: Evidence for an exocrine function of the pulmonary Type II cell. Pharmacologist 16: 299, 1974. (Abstract)

Hook, G. E. R. and DiAugustine, R. P.: Evidence for the secretion of concentric lamellar organelles of the pulmonary Type II cell. International Conference on Lung Diseases, American Thoracic Society & Canadian Thoracic Society, Montreal, Canada, May 1975. (Abstract)

Bhattachargya, S. N., Passero, M. A., DiAugustine, R. P., and Lynn, W. S.: Isolation and characterization of two hydroxyproline-containing glycoproteins from normal animal lung lavage and lamellar bodies. J. Clin. Invest. 1975, in press.

Project No.: Z01 ES 80024-02 PB

1. Pharmacology Branch
- 2.
3. Research Triangle Park, N. C.

PHS-NIH

Individual Project Report

July 1, 1974 through June 30, 1975

PROJECT TITLE: "The Interaction of Water-Insoluble Substrates with Water-Insoluble Enzymes in Aqueous Media"

PREVIOUS SERIAL NUMBER: NIEHS-P-009

PRINCIPAL INVESTIGATORS: G. E. R. Hook, Ph.D. and J. R. Fouts, Ph.D.

OTHER INVESTIGATOR: L. B. Gilmore, B. A.

COOPERATING UNITS: Environmental Toxicology Branch

MAN YEARS

Total	1.0
Professional:	0.5
Other	0.5

PROJECT DESCRIPTION

OBJECTIVES: Most foreign compounds which are acted on by the microsomal mixed-function oxidase system are soluble to only a limited extent in water at physiological pH. The interactions of water-insoluble substrates with these enzymes have been studied extensively in vitro, but generally only with the aid of solubilizing agents designed to bring the substrate into aqueous solution.

Previous investigations associated with this project indicated that the most suitable vehicle for the introduction of highly insoluble compounds such as biphenyl and benzpyrene to microsomal mixed-function oxidases was carboxymethyl cellulose (CMC) in which the substrate was dispensed as a fine suspension.

Using CMC as a carrier for biphenyl, the response of biphenyl 2- and 4-hydroxylase to animal pretreatment with 2,3,7,8-tetrachlorodibenzo-p-dioxin was studied in hepatic and extrahepatic tissues of the rat, rabbit and guinea pig.

METHODS EMPLOYED: Microsomes were isolated from various organs using established methods. Biphenyl hydroxylation was followed using spectrophotofluorometric methods. Other mixed-function oxidases were studied for comparison using spectrophotometric analyses.

MAJOR FINDINGS: Using biphenyl as a model substrate, the response of biphenyl hydroxylases to animal pretreatment with 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) was studied and compared with a number of other mixed-function oxidases.

Significant stimulation of biphenyl 2- and 4- hydroxylase was observed following a single oral dose of only 1 μ g TCDD/kg body weight in both male and female rats. In comparison, benzphetamine N-demethylase was unaffected in female rats but suppressed in male rats. Although biphenyl 2-hydroxylase activities are very low in hepatic microsomes of adult rats, using CMC as carrier in place of the customary Tween 80 (a competitive inhibitor) enabled the kinetics of the reaction to be studied. In the case of biphenyl 2-hydroxylase, TCDD treatment of animals produced no change in the K_m while V_{max} was increased 10-fold. Biphenyl 4-hydroxylase on the other hand responded to TCDD with increases in both K_m and V_{max} . In untreated animals, biphenyl 2-hydroxylation was not observed in any tissue studied in the rabbit or guinea pig, but biphenyl 4-hydroxylase was measurable in all species studied. The response of mixed-function oxidases to TCDD was species and organ dependent, but the inductive effects of TCDD were not confined to the liver. Of all extrahepatic tissues studied, the kidney was the most responsive to the mixed-function oxidase -- inducing effects of TCDD.

Biphenyl in CMC proved to be a model substrate for the study of the inducing effects of TCDD in that the reaction is very sensitive, relatively simple and known to be free from inhibitory effects of the substrate carriers.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE:

Mixed-function oxidases (MFO's) are of vital importance in the toxification and detoxification of foreign compounds. Without these enzyme systems the elimination of lipid-soluble drugs or toxic chemicals from the body would be a very slow process.

Although many mixed-function oxidase reactions can be measured in vitro, most suffer from limitations imposed by the medium in which the substrate is presented to the enzyme. Suspensions of substrate in CMC appear to eliminate these limitations and for the study of microsomal detoxication reactions, biphenyl appears to be a model substrate. The use of biphenyl as substrate seems also to be a very sensitive indicator of alterations in the levels of xenobiotic metabolizing enzymes produced by the highly toxic environmental contaminant, 2,3,7,8-tetrachlorodibenzo-p-dioxin.

Biphenyl may also serve as a prototype substrate for the environmental contaminant PCB's.

PROPOSED COURSE: This project has been terminated.

KEYWORD DESCRIPTORS

Biphenyl hydroxylases; tetrachlorodibenzo-p-dioxin; substrate carrier; microsomes; mixed-function oxidases; hepatic MFO; extrahepatic MFO; kinetics of biphenyl hydroxylase; rabbit; rats; guinea pigs.

PUBLICATIONS

Fowler, B. A., Hook, G. E. R., and Lucier, G. W.: Tetrachlorodibenzo--p-dioxin induction of renal microsomal enzyme systems. 14th Annual Meeting of the Society of Toxicology, Williamsburg, Virginia. March 9-13, 1975. (Abstract)

Hook, G. E. R., Fowler, B. A., Orton, T. C., and Lucier, G. W.: Stimulation and suppression of hepatic and extrahepatic microsomal mixed-function oxidases (MFO's) by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). 14th Annual Meeting of the Society of Toxicology, Williamsburg, Virginia, March 1975. (Abstract)

Hook, G. E. R., Haseman, J. K., and Lucier, G. W.: The induction and suppression of hepatic and extrahepatic microsomal foreign-compound-metabolizing enzymes by 2,3,7,8-tetrachlorodibenzo-p-dioxin. Chem.-Biol. Interactions 10: 199-214, 1975.

Hook, G. E. R., Orton, T. C., Moore, J. A., and Lucier, G. W.: 2,3,7,8-Tetrachlorodibenzo-p-dioxin induced changes in the hydroxylation of biphenyl by rat liver microsomes. Biochem. Pharmacol. 24: 335-340, 1975.

Project No.: Z01 ES 80025-01 PB

1. Pharmacology Branch

2.

3. Research Triangle Park, N. C.

PHS-NIH

Individual Project Report

July 1, 1974 through June 30, 1975

PROJECT TITLE: "Interaction of Aromatic Polycyclic Hydrocarbons with Macromolecules"

PREVIOUS SERIAL NUMBER: None

PRINCIPAL INVESTIGATOR: P. E. Voytek, Ph.D.

OTHER INVESTIGATOR: R. P. DiAugustine, Ph.D.

COOPERATING UNIT: D. M. Jerina, Ph.D., Laboratory of Chemistry, National Institute of Arthritis, Metabolism, and Digestive Diseases

MAN YEARS

Total: 0.8

Professional: 0.8

Other: 0.0

PROJECT DESCRIPTION

OBJECTIVES: To study the interactions of polycyclic aromatic carcinogens with nucleic acids and certain enzymes involved in DNA synthesis. Attempts will be made to isolate and identify carcinogen-bound adducts of DNA and to study further their metabolism, toxicity, influence on cellular replication, and possible interference with DNA synthesis processes. "In vitro" effects of activated polycyclic aromatic carcinogens on DNA polymerase activity will also be studied.

METHODS EMPLOYED: (1) Reaction of epoxides of or microsomal activation products of aromatic polycyclic hydrocarbons with DNA, digestion of DNA and isolation of carcinogen-bound adducts by absorbent chromatography, and (2) DNA polymerase assays.

MAJOR FINDINGS: Project has just been initiated.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE : Little is known about the type of binding that occurs between genetic materials and polycyclic carcinogens. DNA repair studies indicate that damage caused by these carcinogens is probably removed, but the excision products

(carcinogen -- nucleoside adducts) have not been fully identified nor have their biological activities been explored. Studying these products and their effect on cellular processes may supply information leading to a better understanding of the chemical carcinogenesis process.

PROPOSED COURSE: Isolation procedures are presently being developed for carcinogen-nucleoside adducts in order to achieve yields necessary for identification of these and testing for their biological activity.

Reactions between activated carcinogens and DNA polymerase will be started in the near future.

KEYWORD DESCRIPTORS

Carcinogen binding to macromolecules; DNA polymerase; polycyclic hydrocarbons and their epoxides; epoxide -- DNA interactions; carcinogenic -- nucleoside interactions.

1. Pharmacology Branch
- 2.
3. Research Triangle Park, N. C.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

PROJECT TITLE: "DNA Repair Synthesis in Mouse Oocytes and Zygotes, and the Effects of Carcinogens on the Development of the Fertilized Egg 'In Vitro'"

PREVIOUS SERIAL NUMBER: NIEHS-ET-014

PRINCIPAL INVESTIGATORS: K. Ku, Ph.D. and P. E. Voytek, Ph.D.

OTHER INVESTIGATOR: L. A. Moustafa, Ph.D.

COOPERATING UNIT: Environmental Toxicology Branch

MAN YEARS

Total:	1.0
Professional:	1.0
Other:	0.0

PROJECT DESCRIPTION

OBJECTIVES: (1) To establish direct evidence for DNA repair synthesis in secondary oocytes and fertilized eggs and (2) to assess physical and chemical carcinogen effects on the development of the fertilized ova.

METHODS EMPLOYED: Isolation of secondary oocytes and fertilized eggs after superovulation, autoradiography, and culturing of embryos.

MAJOR FINDINGS: UV light-induced DNA synthesis has been shown to occur in secondary mouse oocytes arrested in metaphase II. Occasionally excision repair was also demonstrated in the first polar body. Preliminary results indicate that the zygotes, at the one-cell stage prior to fusion of male and female pronuclei, are sensitive to low doses of UV light as judged by the cells' inability to develop to the blastula stage after treatment.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Man today is exposed to an increasing number of chemical and physical hazards which can result in genetic damage which in turn could lead to cellular mutations, transformation, carcinogenesis, decrease in survival time, and death. Therefore, acquiring knowledge of genetic repair mechanisms

capable of correcting such damage is extremely important. However, little effort has been expended on the repair processes in germ cells, and, in particular, oocytes and fertilized eggs. The importance of DNA repair during early cell stages of development may be greatly magnified over repair found in somatic cells because of the many time sequences in events that may be gene regulated. It would seem that damaged genetic material would have to be rapidly repaired or the development of the embryo would cease or would proceed abnormally. Therefore, the influence of many potential toxic agents, either physical or chemical, on the repair processes during early developmental stages could present hazardous consequences.

PROPOSED COURSE: A manuscript is in preparation describing radiation-induced repair synthesis in secondary mouse oocytes. Effects of radiation on fertilized eggs at the one-cell stage is currently under investigation. Studies of the effects of polycyclic aromatic carcinogens on the development of the fertilized egg from the one cell stage are also in progress.

KEYWORD DESCRIPTORS

DNA repair synthesis in mouse ova; radiation; carcinogens.

Project No.: Z01 ES 80027-01 PB

1. Pharmacology Branch
- 2.
3. Research Triangle Park, N. C.

PHS-NIH

Individual Project Report

July 1, 1974 through June 30, 1975

PROJECT TITLE: "Alterations of the Glucagon Receptor-Adenylate Cyclase System in Hepatomas"

PREVIOUS SERIAL NUMBER: None

PRINCIPAL INVESTIGATOR: R. D. Mirel, M. D.

OTHER INVESTIGATOR: R. P. DiAugustine, Ph.D.

COOPERATING UNITS: Department of Endocrinology, UNC (Dr. Robert Ney)
Dr. Albert Dalton, NCI
Dr. Harold Morris, Howard University and NCI

MAN YEARS

Total:	1.3
Professional:	1.3
Other:	0.0

PROJECT DESCRIPTION

OBJECTIVES: Glucagon is a key hormone in the regulation of glucose homeostasis. Probably its most important site of action is the liver where it stimulates glycogenolysis and gluconeogenesis. Glucagon action appears to be initiated by attachment of glucagon to a specific cell surface receptor which is in some way coupled to membrane-bound adenylate cyclase. Adenylate cyclase is the key enzyme involved in cyclic-AMP production. In several hepatomas induced by carcinogens, there is a loss of adenylate cyclase response to glucagon. One of the main objectives of this project is to elucidate the mechanism of loss of the glucagon-induced adenylate cyclase response in various hepatomas. Is there actual loss of the glucagon receptor or is there masking of the receptor? Or possibly is the defect not in the receptor but in coupling (or transduction) of the receptor to adenylate cyclase.

METHODS EMPLOYED: Tumor transplantation, liquid scintillation, column chromatography, gamma counting, sucrose density gradients, ¹²⁵I-glucagon binding.

MAJOR FINDINGS: We have looked at six transplantable hepatomas originally induced by various chemical carcinogens and found that in the rapidly growing, poorly-differentiated tumors there is loss of glucagon-induced adenylate cyclase response. In the slowly growing, well-differentiated hepatomas, the glucagon response remains intact. It is significant that the fluoride-induced adenylate cyclase activity in the poorly-differentiated tumors is not only intact but is above levels found for normal liver. The epinephrine-induced response (another receptor-mediated adenylate cyclase stimulus) also remains intact. The implications of these findings suggest that the catalytic unit of adenylate cyclase is intact while the glucagon receptor or the coupling of receptor to catalytic unit is abnormal. The above studies have been carried out on two poorly-differentiated mouse hepatomas and two well-differentiated mouse hepatomas from Dr. Albert Dalton at the NCI. Also the studies are being carried out on the Morris rat hepatomas. The studies are being conducted using purified plasma membrane preparations. There is no evidence that the tumors have proteases that are destroying the native glucagon to account for the loss of adenylate cyclase activity. It appears that the glucagon receptor-adenylate-cyclase system is selectively affected in these hepatomas.

In addition to studying adenylate cyclase response in the various hepatomas, we have spent considerable effort improving the adenylate cyclase assay. Current methods employ double-column techniques which are rather laborious. We have developed a single recyclable-column technique with high yields of the enzyme product: radiolabeled cyclic-AMP.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Investigations of alterations of cell surface receptors in neoplastic tissues might lead to the understanding of the molecular events which are responsible for the loss of growth control of cells. Experiments in other laboratories have shown the control of cell proliferation and cytodifferentiation by a factor reacting with the cell surface. Although the initiation of chemical carcinogenesis may depend on the interaction of the carcinogen (or its metabolites) with cellular nucleic acids, the actual loss of growth control, cellular contact inhibition, or metastasis is thought to be due to some epigenetic function or sequence of reactions initiated at the surface of cells. The biochemical or structural events responsible for the loss of glucagon-stimulated adenylate cyclase might bear analogous correlates to those events or macromolecules which are lost or blocked at the cell surface and are important in growth regulation.

PROPOSED COURSE: Currently we are looking at the binding of ¹²⁵I-glucagon to the various hepatoma membranes. The next aspect of the project will utilize various methods for solubilizing and quantifying the glucagon receptor in the hepatomas. Also, we will be trying to "uncover" the receptor in order to understand what phenomena contribute to receptor blockade when there is phenotypic expression of the receptor protein.

KEYWORD DESCRIPTORS

Glucagon; adenylate cyclase; tumor membrane; hepatoma; cyclic-AMP; carcinogenesis; membrane enzymes and receptors.

CONTRACTS

TITLE: "Research on Early Detection of Environmental Agents that Induce Diseases in Children"

CONTRACTOR'S PROJECT DIRECTOR: S. L. Harrison, M.D.

PROJECT OFFICER (NIEHS): J. L. Braun, Ph.D.

DATE CONTRACT INITIATED: June 1, 1972

CURRENT ANNUAL LEVEL: \$52,000

PROJECT DESCRIPTION

OBJECTIVES: The contractor will establish and manage programs at three Medical Centers focusing on the early detection of environmental agents that induce congenital malformations or other diseases in children.

METHODS EMPLOYED: Records are prepared at the three Medical Centers by selected medical students relative to pertinent environmental exposure histories of the child and the parents. These exposure histories, as well as pertinent clinical information, are summarized by the Medical Center Principal Investigator and submitted to the AAP Committee on Environmental Hazards. The Committee reviews the summaries, prepares relevant commentary, and forwards the information to NIEHS as quarterly progress reports.

MAJOR FINDINGS: The Academy coordinated the participation of three pediatric centers in this clinical epidemiologic effort. Reports of detailed studies of approximately 500 cases were reviewed and evaluated under Academy supervision. Follow-up studies of several agents (oral contraceptives, progesterone, aerosols and spray adhesives) were conducted with negative results.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Detection and identification of environmental stressors continues as an important biomedical research endeavor. New leads on possible environmental stressors were generated through this system which provided a mechanism for monitoring the fetus and newborn for evidence of deleterious effects due to various environmental influences.

PROPOSED COURSE: This contract terminated 9/30/74.

KEY WORD DESCRIPTORS: Environment, health, epidemiology, pediatric

BATTELLE NORTHWEST-PACIFIC NORTHWEST LABORATORY, RICHLAND, WASHINGTON
(Interagency Agreement Between NIEHS and AEC, #300A00444)

TITLE: "Study of the Combined Effects of Smoking and Inhalation of Uranium Ore Dust, Radon Daughters and Diesel Oil Exhaust Fumes in Hamsters and Dogs"

CONTRACTOR'S PROJECT DIRECTORS: J. F. Park, Ph.D. and B. O. Stuart, Ph.D.

PROJECT OFFICER (NIEHS): Phillip J. Walsh, Ph.D.

DATE CONTRACT INITIATED: June 1, 1968

CURRENT ANNUAL LEVEL: \$200,000

PROJECT DESCRIPTION:

OBJECTIVES: As a major part of an integrated research program on the effects of exposure to radon daughters on the development of lung cancer in uranium miners, the following animal studies were initiated:

1. Groups of hamsters were exposed to the following combinations:
 - a. radon daughters on room air nuclei,
 - b. radon daughters and carnotite ore dust,
 - c. diesel engine exhaust alone,
 - d. diesel exhaust fumes, radon daughters and ore dust,
 - e. carnotite ore dust.

Concentrations of radon daughters, composition of ore dust and concentrations of other toxic agents in the diesel oil exhaust fumes were carefully controlled. After being exposed to predetermined cumulative dosage levels, the animals were maintained for life for the observation of pathological changes, particularly lung cancer and emphysema.

2. To test specifically the possible synergistic effects of radon daughters and cigarette smoking, studies were initiated on three groups of 18 dogs as described below:
 - a. dogs exposed to radon daughters and carnotite ore dust exposure for four hours per day, 7 days per week at concentrations of 15 nanocuries per liter; with sham smoking;
 - b. dogs exposed to radon daughters and ore as in (a), plus two working shifts of smoking (total of 10 cigarettes per day with resting on alternate hours).

Regular clinical, radiological and radiographic examinations were made during and after the smoking and radon exposures. The dogs, except for one in each group which will be sacrificed for early histopathological studies, were to be held for their life span with observations for pulmonary changes and lung cancer as the end point.

MAJOR FINDINGS: The hamster studies were completed during FY 74.

Fifteen dogs died or were euthanized during this reporting period. Respiratory tract neoplasma have been diagnosed in five different dogs. The histopathological findings for the dogs that have died or were sacrificed have been described in detail in several Battelle progress reports.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: These experiments were designed to determine, in both a short-lived animal, the hamster (life-span 2-3 years), and a long-lived species, the dog (life-span 12-13 years), the possible synergistic effects of chemical agents -- dust, cigarette smoking and diesel exhaust fumes -- operating singly and in concert with exposure to radon daughters upon the production of lung cancer and other lung diseases in animals. To the extent possible in the laboratory the exposure conditions are designed to simulate those to which uranium miners have been exposed, and in whom an increased incidence of lung cancer has been observed.

PROPOSED COURSE: NIEHS support for the dog studies will not continue beyond FY 75.

KEYWORD DESCRIPTORS: radon daughters exposure; lung cancer in uranium miners; smoking and inhalation of uranium ore dust; diesel oil exhaust fumes; combined effects studies in hamsters and dogs

TITLE: "Fate of Heavy Metals and Heavy Metal Complexes in Soils and Plants"

CONTRACTOR'S PROJECT DIRECTOR: R. E. Wildung, Ph.D.

PROJECT OFFICER (NIEHS): Warren T. Piver, Ph.D.

DATE CONTRACT INITIATED: January 1, 1972

CURRENT ANNUAL LEVEL: \$85,000

PROJECT DESCRIPTION

OBJECTIVES: The proposed investigations will aim at determining, by means of radiotracers and advanced chemical analytical techniques, the fate of agriculturally important metals in soils, in soil microorganisms, and in representative grass and broadleafed plants grown on treated soil. The rate and extent of uptake and translocation and the sites of deposition of metallic compounds within the plant will be measured. In microbial and plant tissues in which radioactivity is accumulated, investigations will be directed toward determining the bond types and the chemical forms of the metabolites. Establishment of the deposition locations of metals and metal transformation products will allow both the reaction pathway to be estimated and the rate-limiting reactions to be studied in depth. The results of the fundamental investigations will be utilized to devise recommendations for chemical treatments and soil-crop management practices which may serve as remedial or preventive measures to minimize health hazards arising from the accumulation of metals or their metabolites.

METHODS EMPLOYED: Ritzville soil was thoroughly mixed with labeled and unlabeled isotopes. A split-root (soil/nutrient solution) method was employed to grow soybeans to maturity in the metal containing soils. The plants were cultured in a growth chamber under constant light (14 hr light; 10 hr dark), temperature (27°C light; 20°C dark) and humidity (40-45%). At maturity the plant tops and roots were harvested (133 and 142 days from planting). Plants were separated into cotyledons, roots, stems, leaves, immature bean pods, mature bean pods and mature beans. In all cases the tissues were analyzed for total metal radioisotopes.

With regard to the microbial studies, major emphasis was placed on the characterization of those organisms that exhibited high resistance to metals using various enrichment techniques at different metal concentrations. In the initial studies viable counts were used as a measure of growth, in the more recent work turbidimetric and dry weight measurements have been used.

MAJOR FINDINGS: Initially, methods were developed and applied to the measurement of the optimum uptake and sites of deposition of 17 metals in shoots

from sequential harvests of barley (*Hordeum vulgare*) and soybeans (*Glycine max*) grown in soils containing radiolabelled metals at several concentration levels. In addition, chemical techniques were developed and applied to determination of the solubility, molecular weight distribution, charge and thin-layer chromatographic characteristics of 17 metals in plant tissues. These 17 metals were: silver (Ag), arsenic (As), cadmium (Cd), cobalt (Co), chromium (Cr), copper (Cu), iron (Fe), mercury (Hg), manganese (Mn), molybdenum (Mo), nickel (Ni), lead (Pb), antimony (Sb), tin (Sn), thallium (Tl), tungsten (W), and zinc (Zn). Where radioactivity associated with the metals was below detection levels, metal-ligand exchange techniques were employed to identify metal binding ability of isolated plant components. An ancilliary part of these studies involved measurement of the effects of metals in soil on the synthesis of plant components containing functional amino groups.

Concurrently with the plant studies, investigations were undertaken to determine the biochemical fate of the 17 metals in soils used for plant culture. Initially, methods were developed and applied to the determination of the effects of metals at different concentration levels on changes with time in the numbers of aerobic and anaerobic bacteria, aerobic and anaerobic sporeforming bacteria, actinomycetes and fungi, and on changes in soil respiratory activity. During the course of the microbial studies, changes in the solubility of the metals in soil were determined as a function of incubation time. Detailed investigations of the mechanisms of metal inhibition on microbial processes were also undertaken with selected metals.

From the results of the above-described investigations, 9 metals were selected for intensive study as those exhibiting the most potential for biochemical alteration in the soil and uptake and concentration by plants. These metals were As, Cd, Cr, Hg, Mo, Ni, Ag, Tl, and W.

The split-root technique for plant culture, in combination with autoradiographic and radiochemical analytical techniques used in previous studies, was employed to determine the distribution of the selected metals in the roots, stems, leaves and fruiting bodies of the soybean (*Glycine max*). Plant components containing the highest metal concentrations were subjected to chemical analyses to determine the form of the metal in tissues. Separation procedures previously developed for low molecular weight (<10,000 and <500) components were employed and extended to include detailed characterization and identification of separated metal metabolites. Lower molecular weight substances which were more likely to enter the food chain received initial emphasis; however, techniques were developed for fractionation of higher molecular weight (>10,000) components. Final characterization and identification of components of either the high or low molecular weight fractions were accomplished using a broad range of analytical techniques including thin-layer chromatography, gas chromatography - mass spectrometry, infrared or ultra-violet spectroscopy, and electron-spin resonance.

Using the enrichment techniques previously developed, investigations were undertaken to isolate, identify, and study in vitro microorganisms exhibiting

resistance to metals in the soil employed in plant studies. It is these organisms which have the greatest potential for biochemical modification of metals in soil. Bacteria, fungi, and actinomycetes isolated from soil enrichments which use metals as selective agents will then be evaluated as to their ability to form organometallic complexes from inorganic metal salts.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: A number of avenues exist by which metals may enter the human food chain. The principal direct avenue probably results from using metals in agriculture as pesticides, desiccants or as contaminants of fertilizers. The importance of soil and plants in this pathway has been underscored by the spectre of metal conversion to more soluble and more toxic heavy metal complexes which may subsequently be taken up by plants and thereby enter directly into human food-stuffs. Particular attention should be given to determining the mechanisms for converting inorganic metals to organic complexes and the extent of this conversion. This information should provide a sound basis for estimating the potential hazard arising from the continued use of these materials and may provide the key to remedial measures.

PROPOSED COURSE: On the basis of knowledge accumulated to date, it is proposed that research emphasis be placed on the metals, thallium, chromium, nickel, and cadmium, which are important pollutants associated with energy and food production. Efforts will be directed principally toward (i) biochemical characterization of the form of cellular and exocellular organo-metal complexes produced by microorganisms isolated from soil on the basis of their resistance to metals, (ii) characterization of the soluble organo-metallic complexes in soil using chemical techniques developed in microbial studies, (iii) determination of the plant availability of microbial metabolites in nutrient culture, (iv) measurement of the kinetics of plant uptake of metals from soil, and the extent of mobilization or remobilization to the seed during growth of soybeans to maturity, (v) biochemical characterization of the form of the metal in the plant using techniques similar to those employed in the characterization of metal form in microbial and soil studies, with particular emphasis on those classes of compounds shown to be available to the plant in nutrient culture and metals which exhibit a propensity to localize in the seed.

KEYWORD DESCRIPTORS: heavy metals; soybeans; uptake; distribution in plant components; soil microbial interaction.

BECTON -DICKINSON RESEARCH LABORATORIES
RESEARCH TRIANGLE PARK, N. C.
(N01-ES-4-2164)

TITLE: "The Effects of Chronic Intermittent Inhalation Exposure of Animals to Airborne Pollutants

CONTRACTOR'S PROJECT DIRECTOR: Finis Cavender, Ph.D.

PROJECT OFFICER (NIEHS): Robert T. Drew, Ph.D.

DATE CONTRACT INITIATED: April 29, 1974

CURRENT ANNUAL LEVEL: \$395,000

PROJECT DESCRIPTION

OBJECTIVES: To determine chronic inhalation toxicity of airborne pollutants, alone and in combination with other materials, by inhalation and other routes of administration.

METHODS EMPLOYED: Rodents will be exposed to the airborne materials and the toxicological effects will be assessed using several of the following criteria: morphological changes in selected tissues, alteration of tissue enzyme or biochemical parameters, cardiovascular and respiratory physiological changes, and alteration in normal kinetics of uptake and excretion of various compounds.

A significant amount of time and effort was spent in chamber procurement, installation, and calibration. At the present time, the contractor has operational 12 inhalation chambers each with a volume of about one m³.

MAJOR FINDINGS: The first two compounds chosen for study were sulfuric acid mist and ozone. A series of subacute studies have been completed at various concentrations of sulfuric acid mist and ozone for any synergistic effects. These studies suggest that while effects can be seen from both compounds, there is little or no evidence of a greater-than-additive effect on the parameters measured. Studies are now in progress investigating the effects of particle size, acid concentration per particle and numbers of particles with reference to sulfuric acid mists.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The widespread use of aerosol products, the current emphasis on automobile travel and the recent energy crisis have each added to the already large number of pollutants in the atmosphere. The intramural program of the aerotoxicology section has been developed with the idea of elucidating effects resulting from acute high-level exposure to inhaled materials

singly and in combinations. This contract has been initiated to permit us to compare the effects of acute exposures to those resulting from chronic intermittent exposures.

Exposed animals can be compared to unexposed animals (over a large portion of their lifetime) with respect to many kinds of biological indicators including mortality and growth rates, morphological changes, changes in respiratory and cardiovascular physiological indices and changes in rates of metabolism of the inhaled compounds. Finally, we can study the effects of chronic intermittent exposure to combinations of compounds, testing for greater-than-additive interactions.

Sulfuric acid mist and ozone have been chosen as the first combination of compounds to be investigated because of the presence of these compounds in ambient urban atmospheres. Sulfuric acid mist is also a major by-product of the catalytic converters now being considered for use in control of automobile exhaust emissions.

PROPOSED COURSE: Chronic studies with sulfuric acid mist and ozone will start soon. The second combination of compounds to be investigated will be aluminum-chloride-hydroxide and fluorocarbons.

KEYWORD DESCRIPTORS

Chronic inhalation studies; sulfuric acid mist; ozone; aluminum-chloride-hydroxide; fluorocarbons; toxicity of pollutant mixtures; interactions -- synergism of inhaled pollutants; rodents.

BROOKHAVEN NATIONAL LABORATORY - UPTON, NEW YORK
(Interagency Agreement)

TITLE: "Chemical and Environmental Mutagen Studies Utilizing the Tradescantia Test System"

CONTRACTOR'S PROJECT DIRECTOR: A. H. Sparrow, Ph.D.

PROJECT OFFICER (NIEHS): F. J. de Serres, Ph.D.

DATE CONTRACT INITIATED: June 6, 1972

CURRENT ANNUAL LEVEL: \$168,000

PROJECT DESCRIPTION

OBJECTIVES: The objectives of the proposed research are: (a) to demonstrate the usefulness of and further develop the Tradescantia somatic mutation test system as a quantitative monitor in environments containing known or suspected mutagens; (b) to use special genetic clones of Tradescantia to establish the relative mutagenicity of a limited number of mutagens (primarily in gaseous form), as to magnitude of effect, specificity of action and possibility of reversion; (c) to look for evidence of genetic repair of DNA injury produced by chemical mutagens and to look for possible threshold levels below which induced mutation rate is negligible and (d) to determine in greater detail the time and dose response for several loci for one selected gaseous mutagen, comparing these with presently available radiation data and with results observed in animal cells.

METHODS EMPLOYED: Two different clones of Tradescantia (clone 02 and clone 4430), which are heterozygous for a flower petal color mutation were used to determine the mutagenicity of exposure to various concentrations of SO_2 , NO_2 and ozone. Refinements in chemical mutagen exposure techniques were made to establish 1,3-dibromoethanol (DBE) as the mutagen standard in these studies rather than ethylmethane sulfonate (EMS). Plant breeding and new clone selection has also been continued to obtain more sensitive clones which might provide better indicators of weak mutagens and chronic levels of exposure. New clones with a wider range of spontaneous mutation rates at each of three loci are being used along with existing mutable clones to develop methods for studying repair.

MAJOR FINDINGS: New data were acquired in relation to mutagen concentration and duration of exposure for 1,2-dibromoethane (DBE) and to exploring the concept of time x concentration. DBE has also been used to obtain base-line data for exposures extending beyond one cell cycle time. Longer exposures, including chronic exposures, are planned for the various atmospheric oxidants such as ozone, SO_2 , NO_2 , etc., but only base-line data from DBE exposures up to 18 hours are currently available.

As new chemicals and new clones became available, additional exposures were made. Preliminary data for Freon-12 are shown in comparison with data for

other representative gaseous chemicals such as DBE, trimethyl phosphate, SO₂ and NO₂. Exposures have been made to another fluorocarbon, Freon-22, but data are not yet available.

Several new hybrids between clones 02 and I. subacaulis are now available and preliminary x-ray dose response data indicate that they are less sensitive than clones 02 and 4430.

Other areas include temperature effects on mutation rate, possible repair mechanisms as evidenced by split dose exposures (x-ray), comparisons of DBE and EMS exposures of normal and mutable clones of Tradescantia, isotope labeling of stamen hair cells as a technique for determining penetration and/or quantitative uptake of chemical mutagens and pollen tube culture as a technique for studying mutagen-induced chromosome aberrations.

Considerations of the pollen tube technique and a study of factors influencing background mutation level introduce the need for clean-room facilities. Clean air studies were made with regard to determining availability and costs of air purifying equipment for plant growth rooms, culture rooms and portable exposure chambers required to support research along these lines.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: This contract should provide a badly needed assay for mutagenic airborne environmental chemicals. It is extremely sensitive to ionizing radiation, and the exploratory work with selected chemical mutagens during this past year suggests that it may be able to detect concentrations as low as 10 ppm. This assay system should provide a meaningful assay for the production of gene mutations and relates to the Institute's program to develop sensitive assay systems to detect the mutagenic activity of environmental chemicals.

PROPOSED COURSE: At the conclusion of the present course of oxidant exposures, work will focus on determining the relative mutagenicity of specified industrial compounds using DBE as the standard. Plans are also being made to develop a mobile laboratory to make it possible to assay airborne environmental pollutants at field stations in the New York City area where NERC-EPA is measuring ozone, SO₂ and NO₂ levels.

KEYWORD DESCRIPTORS: Somatic mutation test system, genetic repair of DNA injury, thresholds, chemical mutagens.

TITLE: "Studies of the Effects of Environmental Components on Reproduction and Ontogenesis"

CONTRACTOR'S PROJECT DIRECTOR: B. A. Schwetz, D.V.M., Ph.D.

PROJECT OFFICER (NIEHS): Robert E. Staples, Ph.D.

DATE CONTRACT INITIATED: February 5, 1973

CURRENT ANNUAL LEVEL: \$297,615

PROJECT DESCRIPTION

OBJECTIVES: (1) To determine the teratogenic potential in animals of environmental components when administered singly or in combination, (2) to determine species differences in response to teratogens and to study the etiology of such differences and (3) to investigate the mechanism of action of teratogens and to improve experimental testing and extrapolation to man.

METHODS EMPLOYED: In general, the screening approach outlined by the World Health Organization (Technical Report No. 364, 1967) is being followed with certain modifications to alleviate some of the problems encountered by private industry during the testing of potential therapeutic agents.

MAJOR FINDINGS: Carbon monoxide was administered¹ at 250 ppm by inhalation to mice and rabbits. A teratogenic response was not detected after examination of the fetuses before term for external, visceral, or skeletal alterations.

Phenobarbital was not teratogenic when administered by gavage at 60 and 30 mg/kg/day from Days 4-15 (mice) and Days 4-18 (rabbits), respectively. These dose levels were high enough to stimulate microsomal enzymes as measured by reduced hexobarbital sleeping times in both species.

Piperonyl butoxide, an inhibition of hepatic microsomal enzyme activity and an insecticide synergist, was administered¹ to mice, by gavage, at 300 mg/kg/day. A teratogenic response was not noted even when piperonyl butoxide was administered in combination¹ with Dichlorvos (60 mg/kg/day) and an increase in toxicity was not noted. This finding suggests that the increased incidence of cleft palates observed in mice given SKF-525A in combination with Dichlorvos was not likely due to inhibition of Dichlorvos metabolism but to some other activity of SKF-525A.

Carbaryl was administered to mice for 10 days and to rabbits for 13 days by gavage to determine the maximum tolerated dose (MTD) for use in studies designed for determining teratogenic potential. The MTD established was 100 mg/kg/day in mice and 200 mg/kg/day in rabbits. The MTD for mice when incorporated in their diet was 1200 mg/kg/day.

¹ Agents were administered during the period of major organogenesis. This period was considered to be Days 6 through 15 of gestation for the mouse and Days 6 through 18 of gestation for the rabbit.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The etiology of about 60% of human congenital malformations is currently unknown. It is likely that chemical and physical factors in man's environment, acting either singly or in combination, might account for an important fraction of human birth defects.

This contract attempts to carefully evaluate the teratogenic potential of selected environmental factors either alone or in various combinations. In addition, attempts will be made to define basic teratogenic mechanisms which will allow for better predictive extrapolation from experimental animals to man.

PROPOSED COURSE: Carbaryl will be studied for teratogenic potential in rabbits and mice by being administered alone and in combination with other environmental factors e.g., low protein diet, piperonyl butoxide, carbon monoxide, pheno-barbital, alcohol.

Dichlorvos will be administered to mice and perhaps rabbits by inhalation alone and in combination with carbon monoxide; carbon monoxide also will be studied in combination with alcohol.

KEYWORD DESCRIPTORS: teratogenic potential; environmental components; teratogens; carbon monoxide; inhalation; mice; rabbits; gavage; Dichlorvos; cleft palates; SKF-525A; carbaryl; low protein diet; alcohol.

TITLE: "Development of Microwave Instrumentation"

CONTRACTOR'S PROJECT DIRECTOR: William T. Joines, Ph.D.

PROJECT OFFICER (NIEHS): Donald I. McRee, Ph.D.

DATE CONTRACT INITIATED: June 1, 1973

CURRENT ANNUAL LEVEL: \$15,267

PROJECT DESCRIPTION

OBJECTIVES: The proposed work of this contract is threefold: (1) Study the electrical dipole model of the neural membrane and its implications, and to determine the unknown parameters in the interaction of the dipole model with microwave radiation, (2) Develop microwave instrumentation and methods to investigate microwave-neuron interactions over the 0.2 to 3.0 GHz frequency band, (3) Develop the instrumentation and dosimetry of a more accurate method of determining the microwave power absorbed by a neuron.

MAJOR FINDINGS: Using computer oriented skull models, radiation dosage levels within the skull were investigated for a range of microwave frequencies. With 10 mW/cm^2 of incident radiation at 2.1 GHz, the power density reaches a maximum of about 20 mW/cm^3 near the center of the brain for a model of 7 cm radius. These calculated power density levels were used as a dosage guide in irradiating live neurons positioned inside a strip transmission line. It was also shown that the homogeneous sphere model of the skull is in error between 1 and 3 GHz; thus this model should not be used to determine potential hazards over this frequency range.

Two equivalent circuits for modeling microwave-neuron interactions were investigated. For the perfect rectifier equivalent circuit with 10 mW/cm^2 incident radiation, the induced transmembrane potential is 27.5 volts, 10,000 times larger than the voltage necessary to influence neuron functioning under the employed experimental set up. For the dipole model equivalent circuit, under certain assumptions, depolarization of the neural membrane can exceed the threshold value necessary to fire the neuron. Sample calculations predict that microwave pulses 8 to 80 microseconds wide, applied at a rate less than about 100 pulses per second, should be most effective for nerve stimulation. The theory predicts a minimum power level, and perhaps a maximum power level for optimum microwave-neuron interaction. These predictions are shown to be consistent with results of other investigators. Measurement technique and instrumentation were developed to determine the absorbed dose of a neuron placed in a stripline exposure unit. The measured values agreed with the theoretical values in the frequency range of 1 to 3 GHz.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: This contract work on isolated cells and organs complement the present work in progress at NIEHS, where experiments on the neurological effects of microwaves are being conducted. Work at NIEHS concerns effects in mammalian systems,

while the Duke work concerns effects in isolated ganglia of cold blooded animals.

PROPOSED COURSE: This contract was terminated September 30, 1974.

KEYWORD DESCRIPTORS: Microwave dosimetry, instrumentation, stripline, microwave-neuron interaction.

PUBLICATIONS

Joines, W.T., Spiegel, R.J.: A semiclassical theory for nerve excitation by a low intensity electromagnetic field. Bulletin of Mathematical Biology, Vol. 35, Oct., 1973.

Joines, W.T. and Spiegel, R.J.: Resonance absorption of microwaves by the human skull, IEEE Trans. on Biomedical Engineering, Vol. BME-21, Jan. 1974.

Wachtel, H., Season, R., Joines, W.T.: Effects of low intensity microwaves on isolated neurons, Annals of the New York Academy of Sciences, Vol 247, Feb. 1975, pp. 46-62.

FLORIDA STATE UNIVERSITY, DEPARTMENT OF OCEANOGRAPHY
TALLAHASSEE, FLORIDA 32306
NO1-ES-5-2113

TITLE: "Studies on the Physical Characterization of the Respiratory Irritant Associated with Gymnodinium breve Davis Blooms, Red Tide, on the West Coast of Florida"

CONTRACTOR'S PROJECT DIRECTOR: John W. Winchester, Ph.D.

PROJECT OFFICER (NIEHS): Robert T. Drew, Ph.D.

DATE CONTRACT INITIATED: January 1, 1975

CURRENT ANNUAL LEVEL: \$13,348 (January 1 - June 30, 1975)

PROJECT DESCRIPTION

OBJECTIVES: It has been known for some time that an airborne human respiratory irritant is associated with outbreaks of Florida's gulf coast red tides (Gymnodinium breve). The object of these studies is to collect atmospheric samples and to compare samples taken in the presence and the absence of a red tide. Size selective aerosol samplers will enable comparison of specific size fractions for: presence of marine microorganisms, elemental analysis, and investigations of the toxicity of each size fraction.

METHODS EMPLOYED: Cascade impactors will be used to sample the atmosphere. Elemental analysis will be accomplished by proton-induced X-ray emission analysis. Identification of microorganisms will be performed with the use of a scanning electron microscope.

MAJOR FINDINGS: The methodology and logistics of sampling for red tide aerosols have been developed and tried out during a background run. A mini-ocean environment has been inoculated with Gymnodinium breve and attempts to generate an aerosol from this environment are in progress. The collection of background information has confirmed that all the sampling methods are feasible and can be adapted to field situations.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The gulf coast of Florida has been subjected to occasional outbreaks of red tides caused by the dinoflagellate Gymnodinium breve for at least a century. The most notable effect of these red tides has been massive fish kills. Recently, however, people exposed to ocean spray aerosols in the presence of red tide have experienced acute respiratory distress. The research in this contract is part of an overall task designed to: identify and characterize the respiratory irritant, develop animal models to study the respiratory response, investigate the mechanism of irritant action, develop methods of ameliorating the human respiratory

response and conduct epidemiological studies of both humans and animals in red tide areas.

PROPOSED COURSE: It is proposed to extend this project at approximately the same level of effort for at least one additional year.

KEYWORD DESCRIPTORS

Red tide; Gymnodinium breve; biological respiratory irritants; aerosol.

TITLE: "Development of a New Mouse Strain to Maximize the Sensitivity of a Point Mutation Assay"

CONTRACTOR'S PROJECT DIRECTOR: Thomas H. Roderick, Ph.D.

PROJECT OFFICER (NIEHS): Lawrence R. Valcovic, Ph.D.

DATE CONTRACT INITIATED: February 19, 1974

CURRENT ANNUAL LEVEL: \$33,800

PROJECT DESCRIPTION

OBJECTIVES: The sensitivity of a point mutational assay system is directly related to the number of loci that can be tested in each individual. In the present system in our laboratory, nine loci are sampled. Other inbred strains of mice possess enzyme variants which can possibly be incorporated into one of our present strains. This contract seeks to fully identify these additional variants (at least 13) and then by repeated backcrossing to incorporate them into the C57BL/6J genome.

METHODS EMPLOYED: Starch gel electrophoresis is used to determine whether a strain carries an allele that is not found in either the DBA/2J or C57BL/6J strains. When such a variant is found, the strain is backcrossed repeatedly to C57BL/6J for 8-10 generations to develop a new strain which will be homozygous with C57BL/6J. Electrophoresis tests will be performed on progeny in each generation to insure that the allelic differences already present in the C57BL/6J line are maintained.

MAJOR FINDINGS: Several strains carrying new variant alleles have been crossed to the C57BL/6J strain. Most lines have proceeded through the fourth backcross generation with electrophoretic verification of the incorporation of the variant alleles.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: While many compounds in the environment have been shown to cause point mutations in lower organisms, fewer compounds have been tested in mammals due to the lack of a feasible test system. Also, a program to monitor the human population for the detection of electrophoretic mobility mutations is in the developmental stages. However, no comparable study has been conducted with any laboratory mammal and hence, there is no base-line data for known mutagens for this type of mutational event. In an attempt to elucidate these areas, we are presently using two mouse strains that have nine different isozymes which can be detected electrophoretically. Since maximal sensitivity depends on polymorphic loci, it is desirable to increase the number of enzyme differences between the two strains. There are at least 13 other allelic differences which are present in various other strains or stocks of mice. If these were

incorporated into one of our strains, this would greatly increase the sensitivity of the system with a minimal increase in efforts. The strain will be made available for mutagenesis testing of environmental pollutants, carcinogens and drugs to the scientists in the Environmental Mutagenesis Branch.

PROPOSED COURSE: The offspring obtained from crosses will be analyzed for particular loci and those carrying the desired alleles will be backcrossed to C57BL/6J. The procedure will be carried for 8-10 generations.

KEYWORD DESCRIPTORS: Point mutations, mammals, electrophoresis, allelic differences, isozymes, mutagenicity testing.

TITLE: "Development of a System for Detecting Lethal Mutations in Mice"

CONTRACTOR'S PROJECT DIRECTOR: Thomas H. Roderick, Ph.D.

PROJECT OFFICER (NIEHS): William Sheridan, Ph.D.

DATE CONTRACT INITIATED: January 15, 1974

CURRENT ANNUAL LEVEL: \$50,000

PROJECT DESCRIPTION

OBJECTIVES: At present no efficient test system exists for detection of point mutations in mammals. The aim of the contract is to develop strains of mice, carrying marked inversions, which may be used in studies of induction of point mutations. Theoretically these tests will be comparable to the system presently used in *Drosophila*. The prime goal is to induce inversions in as many chromosomes of the mouse as possible and combine them in a number of strains to be used as tester stocks.

METHODS EMPLOYED: The contractor induces chromosome breaks and inversions by means of radiation and/or chemical agents. He mates the treated animals and in the F_1 does cytological analysis of one testis of each individual male. If a significant increase in anaphase bridges appears, this is taken as an indication that the animal may carry an inversion, and he is mated to get offspring for further study. Following cytological and linkage studies to characterize the aberration, decision is made as to the utility of the inversion and appropriate marker genes are introduced into the stock.

MAJOR FINDINGS: The contractor has induced and recovered 19 inversions. Of these, five have been thoroughly characterized for linkage and chromosomal morphology. One of the inversions has been successfully utilized in tests to detect induced recessive lethal mutations.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Recessive lethal mutations are known to exist at high frequencies in human populations. Many environmental compounds are known to be mutagenic and capable of causing both chromosome aberrations and point mutations. With an efficient animal model to study the inductions of such mutation, more accurate estimates can be made of the risks such compounds imply to the human population.

PROPOSED COURSE: The estimated length of the contract is five years. Efforts will continue to induce and characterize further inversions. It is proposed to combine inversions with Robertsonian Translocations to increase the effectiveness of the system.

KEYWORD DESCRIPTORS: Mice, marked inversions, point mutations, inversions, chemical agents, anaphase bridges, recessive lethal mutations, Robertsonian Translocations.

TITLE: "The Effects of Dietary Deficiency on the Sensitivity to Mutagenic Treatment"

CONTRACTOR'S PROJECT DIRECTOR: Billy W. Geer, Ph.D.

PROJECT OFFICER (NIEHS): William Sheridan, Ph.D.

DATE CONTRACT INITIATED: May 1, 1975

CURRENT ANNUAL LEVEL: \$44,583

PROJECT DESCRIPTION

OBJECTIVES: In investigations using Drosophila melanogaster cultured on synthetic media deficient in Vitamin E, the frequency of chromosomal effects which were induced by irradiation was increased compared with treated cultures raised on non-deficient media. It is our purpose to investigate whether or not dietary deficiency might change the overall physiological state of the organism making it more susceptible to the actions of mutagenic agents.

METHODS EMPLOYED: Wild-type Drosophila will be raised on a complete, defined, synthetic, germ-free medium. Subcultures will be placed on eleven (11) different media from which specific vitamins and minerals have been singly eliminated or depleted. Flies on deficient media and flies on whole medium will be exposed to similar doses of a known potent chemical mutagen. The frequencies of recessive lethal mutations, dominant lethal mutations and chromosomal aberrations (translocations) will be determined. Comparisons between the treated groups and with concomitant control groups will be made in order to study any differences in the occurrence of induced genetic damage related to the diet.

MAJOR FINDINGS: The effective date of this contract is May 1, 1975.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: A greater part of the human population on a world-wide basis is known to be subjected to nutritional insufficiency at the present time. At the same time it is known that there are present in the human environment compounds which are mutagenic. Knowledge of the existence of effects of dietary deficiency on sensitivity to mutation induction will be a necessary consideration in attempts to extrapolate from information derived from laboratory animals in estimating risks to the human population.

PROPOSED COURSE: The estimated length of the contract is two years.

KEYWORD DESCRIPTORS: Dietary deficiency, Drosophila, recessive lethal mutations, chromosomal aberrations, chemical mutagen, synthetic medium.

TITLE: "Interaction of Noise and Ototoxic Drugs on Hearing Loss in Animals"

CONTRACTOR'S PROJECT DIRECTORS: Merle Lawrence, Ph.D. and Joseph Hawkins, Ph.D.

PROJECT OFFICER (NIEHS): Reginald O. Cook

ASSISTANT PROJECT MONITOR: Philip W. Albro, Ph.D.

DATE CONTRACT INITIATED: June 15, 1972

CURRENT ANNUAL LEVEL: \$100,000

PROJECT DESCRIPTION

OBJECTIVES : To determine the extent and the nature of the interaction between noise and ototoxic drugs in experimental animals, and to study the biochemical mechanisms involved in such losses. More particularly, objectives are:

1. Prove or disprove the involvement of phospholipid metabolism in the destruction of inner ear tissue and function under the influence of neomycin.
2. Investigate the biochemical changes induced by noise exposure (pO_2 , ATP, Na^+/K^+ , protein and lipid metabolism).
3. Investigate the biochemical changes under the influence of a combined noise and drug treatment. Monitor and correlate ATPase as a function of time after treatment.
4. Determine the mechanism of action of neomycin on polyphosphoinositide metabolism.

MAJOR FINDINGS: The biochemical mechanism of neomycin ototoxicity was investigated in guinea pigs in vivo and in vitro. In vivo, the incorporation of ^{32}P -orthophosphate into the phosphoinositide lipids of inner ear tissues was measured in normal and in neomycin-treated guinea pigs. These lipids are believed to be involved in the control of membrane permeability. There was a decrease of labeling of phosphatidylinositol diphosphate in the organ of Corti and stria vascularis following three weeks of daily injections of neomycin (100 mg/kg body weight). In homogenates of organ of Corti and stria vascularis the hydrolysis of phosphatidylinositol diphosphate was blocked and the binding of ^{45}Ca was inhibited in the presence of neomycin.

Since neomycin is both ototoxic and nephrotoxic, the biochemical studies were extended to investigate phospholipid labeling in guinea pig kidney, liver and brain. Twenty to forty minutes after intraperitoneal injection of ^{32}P , the most highly labeled lipids in the kidney are phosphatidylinositol phosphate and diphosphate. Treatment of animals with neomycin for six days decreases labeling of these lipids. Such decrease is not seen in ATP or the other labeled lipids (phosphatidyl choline, ethanolamine, inositol and serine) and is not observed in liver and brain. In vitro experiments with kidney

subcellular fractions demonstrate inhibition by neomycin of the hydrolysis of phosphatidyl inositol phosphate and diphosphate.

These studies cannot establish yet a causal relationship between the neomycin toxicity and its inhibition of phosphoinositide metabolism. Other contract studies show, however, at least a parallel effect of the drug on cochlear microphonics and lipid metabolism.

The neomycin/lipid interaction has been studied in vitro not only in inner ear but also kidney and brain subcellular fractions. Such experiments demonstrated that the effect on phosphoinositide metabolism is exerted by neomycin and those related antibiotics that are toxic to the cochlea and the kidney but not by other aminoglycoside antibiotics lacking this toxicity.

It is suggested that at least part of the ototoxic action of neomycin is mediated by an inhibition of polyphosphoinositide turnover, possibly by direct binding of the antibiotic to these lipids.

Studies of adenylyl cyclase have been the most successful. Cellular functions regulated by adenylyl cyclase include protein synthesis and secretion, membrane permeability, and active transport of ions. Cochlear adenylyl cyclase have been histochemically as well as in tissue homogenates in vitro with the specific substrate adenylyl imido-diphosphate (AMP-PNP). Adenylyl cyclase activity was demonstrated in the plasma membranes of the inner sulcus cells, the interdental cell of the limbus epithelium, and in the infolding of the mesothelium cells covering the perilymphatic surface of the limbus. Studies of drug or noise effects have not yet been undertaken.

The biochemical investigations include studies of ^{32}P -incorporation into lipids and amino acid incorporation into protein. Techniques for both have been established.

Two weeks after kanamycin treatment, injury to the cochlea was assessed in terms of reduction in the microphonic potential (CM) recorded at the round window and hair cell loss as evaluated in surface preparations. Pathological changes were greatest when noise exposure was concurrent with kanamycin treatment or preceded it by only one week. Noise exposure alone and other sequences, with and without an intervening week of rest, produced lesser degrees of injury. The results are compatible with the hypothesis that noise exposure affects the hemato-labyrinthine barrier, causing a more rapid accumulation of the antibiotic in the inner ear fluids and tissues or a reduced rate of clearance from them.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The burden from excessive noise in the general population is extensive and even more so among industrial workers. It now appears that certain drugs may greatly potentiate this noise damage at low levels of noise. This contract has delineated suspected synergisms and investigated the biochemical mechanisms by which such damage occurs.

PROPOSED COURSE: This contract is set to expire June 30, 1975 and is not being renewed.

KEYWORD DESCRIPTORS: Hearing, noise ototoxic drugs, synergism, biochemistry, electrophysiology.

PUBLICATIONS

Kerr, T.P., and Schacht, J.: Adenyl cyclase in the Guinea pig cochlea. J. Acoust. Soc. Amer. 57: 61, 1975.

Kerr, T.P., and Schacht, J.: Cytochemical localization of adenyl cyclase and adenosine triphosphatase activity in the epithelium of the spiral limbus and inner sulcus. 18th Annual Meeting Committee for Research in Otolaryngology, Dallas, Texas, October, 1974.

Marques, D.M., Clark, C.S., and Hawkins, J.E., Jr.: Potentiation of cochlear injury by noise and ototoxic antibiotics in guinea pigs. J. Acoust. Soc. Amer. 57: 60, 1975.

Nuttal, A.L., Marques, D.M., Stockhorst, E., and Schacht, J.: Neomycin effects on cochlear microphonics and phospholipid metabolism. J. Acoust. Soc. Amer. 57: 60, 1975.

Orsulakova, A., Stockhorst, E., and Schacht, J.: Effect of neomycin on phosphoinositide labeling and calcium binding in guinea pig inner ear tissues in vivo and in vitro. J. Neurochem., in press.

Schacht, J.: Action of aminoglycoside antibiotics on phosphoinositide metabolism in guinea pig brain and inner ear tissues. Trans Am. Soc. Neurochem. 5: 66, 1974.

Schacht, J.: Biochemistry of neomycin ototoxicity. J. Acoust. Soc. Amer., in press.

Schacht, J.: Biochemistry of neomycin ototoxicity: Inhibition of calcium binding and turnover of membrane polyphosphoinositides. J. Acoust. Soc. Amer. 56: 20, 1974.

Schacht, J.: Interaction of neomycin with phospholipid metabolism: A mechanism of its ototoxicity? 107th Annual Meeting American Otolological Society, Palm Beach, Florida, April, 1974.

Schacht, J.: Interaction of neomycin with phosphoinositide metabolism in guinea pig inner ear and brain tissues. Ann. Otol. 83: 613-618, 1974.

Schacht, J.: Interaction of neomycin with phosphoinositide metabolism in guinea pig liver, ear and brain tissues. Trans. Amer. Otol. Soc. 62: 93-99, 1974.

Schacht, J.: Neomycin ototoxicity: Inhibition of calcium binding and turnover of membrane polyphosphoinositides. 11th Congress of Inner Ear Biology, Wurzburg, Germany, September, 1974.

Schacht, J.: Ototoxizität von aminoglykosiden und phospholipid stoffwechsel im innenohr. 10th Congress of Inner Ear Biology, Graz, Austria, September, 1974.

Schacht, J., Orsulakova, A., and Grossman, S.D.: Inhibition by neomycin of polyphosphoinositide turnover and calcium binding in guinea pig inner ear tissues. Trans. Amer. Soc. Neurochem. 6: 185, 1975.

LOVELACE FOUNDATION FOR MEDICAL EDUCATION AND RESEARCH
ALBUQUERQUE, NEW MEXICO
(Interagency Agreement Between NIEHS and AEC)

TITLE: "Respiratory Tract Deposition Models"

CONTRACTOR'S PROJECT DIRECTOR: Otto G. Raabe, Ph.D.

PROJECT OFFICER (NIEHS): Phillip J. Walsh, Ph.D.

DATE CONTRACT INITIATED: November 11, 1971

CURRENT ANNUAL LEVEL: \$150,000

PROJECT DESCRIPTION

OBJECTIVES: The major objectives of this research are:

- (1) Construction of physical models of the respiratory tracts of man and several animal species;
- (2) Measurement of the deposition of aerosols as small as 0.01 micron diameter in the models;
- (3) Determination of deposition of selected particle sizes in animals and in man if practical;
- (4) Development of a theoretical model of deposition in the lung based on the above results;
- (5) Field evaluation of the physical-theoretical model.

MAJOR FINDINGS: The morphometric data collected in Stage 1 of the project are now being subjected to extensive statistical analysis as part of Stage 4 in Development of Theoretical Models of the lungs of the four species; hamster, rat, dog and man.

A Monte Carlo sampling technique has been developed and coded which selects terminal paths at random. Two thousand random paths for each lobe of the human lung have been sampled and frequency distributions of terminal generation numbers, total path lengths, terminal bronchiole lengths and terminal bronchiole diameters have been prepared. Obvious differences exist from lobe to lobe with the lower lobes having longer paths and more generations to termination. Furthermore, terminal bronchioles are larger in the left lung than in the right lung.

Generations to termination appears to be log normally distributed and currently these distributions are being fitted to smoothed distributions by non-linear least squares. Within a lobe, the distribution of path lengths are bimodal or trimodal indicating the presence of major and minor pathways. Terminal bronchiole lengths and diameters also appear to be log normally distributed with a high variance of lengths but a small variance of diameters.

A stratified Monte Carlo sampler has also been developed which selects paths at random to generate distributions of lengths, diameters, flow angles and gravity angles for each generation down. These families of distributions will then contain the size dependencies of lung geometry and random samples from each generation will be used to model particle deposition.

The development of the physical models of the respiratory tract has progressed to the testing of various types of models of respiratory tract compartments. The concept of using interchangeable modules for these model compartments allows their testing together, individually, or in any combination. An important concept developed in this work is the use of inter-module aerosol samples for determination of particle deposition in the various compartments rather than attempting to measure the actual deposition. This principle will greatly simplify field studies with physical models.

Deposition studies with hamsters and rats have been completed with monodisperse aerosols of ^{169}Yb -labeled aluminosilicate particles formed by heat fusion of clay aerosols containing ion-exchanged ^{169}Yb . The data has been completely analysed and a report is in preparation. Although quite variable, results show that the minimum pulmonary deposition occurred with the $1.14\text{ }\mu\text{m AD}$ aerosol and higher pulmonary deposition fractions were observed for both larger and smaller particles. For human a local minimum for pulmonary deposition is believed to occur at $0.6\text{ }\mu\text{m AD}$.

The inhalation exposure equipment for the beagle dog deposition studies and the longitudinal scanning equipment for measurement of regional deposition have been assembled and tested. The exposure system incorporates a new principle, concurrent flow spirometry, for maintenance of uniform aerosol concentrations and measurement of inspiratory and expiratory volumetric flow rates.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Improved respiratory tract deposition models are needed to develop insight into the manner in which the comparative morphology of the lung influences the deposition of inhaled particles in experimental animals and man. In particular, such a model would aid in establishing quantitative dose-response relationships laboratory experiments and would effect more meaningful extrapolations to human exposure situations.

PROPOSED COURSE: This project will continue as outlined.

KEYWORD DESCRIPTORS: Respiratory tract, deposition, aerosols, model, animal, man, theoretical model.

PUBLICATIONS

Phalen, R.F., Yeh, H.C., Raabe, O.G. and Velasquez, D.J.: Casting the lungs in situ. The Anatomical Record. 177: No. 2, 255-263, 1973.

Yeh, H.C.: Use of a heat transfer analogy for a mathematical model of respiratory tract deposition. Bull. Math. Biology 36: 106-116, 1974.

Phalen, R.F., Yeh, H.C., Raabe, O.G., Hulbert, A.J. and Crain, C.R.: Comparative airway anatomy in the human, beagle dog, rat and hamster and implications in inhaled particles. Health Physics 27: 6, 1974.

Phalen, R.F. and Raabe, O.G.: Aerosol particle size as a factor in pulmonary toxicity. Proceedings of the Fifth Annual Conference on Environmental Toxicology (In press).

Yeh, H.C., Hulbert, A.J., Phalen, R.F., Velasquez, D.J. and Harris, T.D.: A stereo X-ray method for evaluating changes in airway dimensions during lung casting (In press).

TITLE: "An Appraisal of Environmental Exposure to Heavy Metals"

CONTRACTOR'S PROJECT DIRECTOR: Ivan C. Smith, Ph.D.

PROJECT OFFICER (NIEHS): Warren T. Piver, Ph.D.

DATE CONTRACT INITIATED: April 17, 1972

CURRENT ANNUAL LEVEL: \$56,000

PROJECT DESCRIPTION

OBJECTIVES: To explore environmental contamination with specific metals which may be hazardous to health. To search the literature and other reports and to seek expert opinions from industry in order to compile these data on potential hazards of specific metals. Special emphasis is placed on determination of the physical parameters and chemical properties, valency, reactivity, etc., as the metal compound makes contact with man. The information is to be put together in a document on each of the metals of specific interest.

MAJOR FINDINGS: Within the past year, a report on palladium has been produced. This document has attracted major attention since a platinum-palladium alloy is being used as the active surface in the catalytic muffler. This device is being used to achieve ambient air quality standards for hydrocarbons and CO in mobile sources. These reports have a special significance because they attempt to define the chemical state of the metal in the environment as a result of its release due to intended use or as a contaminant in air and water. The form of the metal and the route of exposure have an important influence on its hazard potential. This type of information distinguishes these reports from the many other reports being produced on metals.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: This surveillance is essential to stay abreast of changes in environmental contamination by certain industrial or communal practices involving different metals or different forms of metals, or their complexes. The information will determine whether research on the health effects of the compound in question should be undertaken and in which direction it should go.

PROPOSED COURSE: The information is being gathered for a number of metals of importance from the toxicologic viewpoint. A report on silver is forthcoming. This is to be followed by reports on zirconium and thallium.

KEYWORD DESCRIPTORS: heavy metals; sources of environmental release; uses; distribution; metal toxicity.

TITLE: "Teratology of Thioxanthone Derivatives"

CONTRACTOR'S PROJECT DIRECTOR: Mr. Jan L. Minor

PROJECT OFFICER (NIEHS): Dr. John A. Moore

DATE CONTRACT INITIATED: April 15, 1974

CURRENT ANNUAL LEVEL: \$52,054

PROJECT DESCRIPTION

OBJECTIVES: To collect teratological data on the teratogenic thioxanthone derivative and selected analogs.

METHODS EMPLOYED: Classical teratology examination of mice.

MAJOR FINDINGS: The contractor has determined what effects, at what dose range, during which period of organogenesis, the analogs may have on the dam and developing mouse embryo. Six analogs were studied. Following determination of effective dose ranges, type of effect, and susceptible stage of organogenesis, in the initial experiment, the contractor conducted a second or definitive experiment to define, with greater precision, the dose-effect relationship involved and characterize the type of response produced during the susceptible stage of embryogenesis.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: This class of compounds have been demonstrated to be effective in the treatment of schistosomiasis. Mutagenic and teratogenic responses have also been observed in some instances. If analogs could be found that are not teratogenic and continue to possess desired antischistosomacidal properties, medicine would be well served for one to pursue the development of these chemicals.

PROPOSED COURSE: The data which resulted from these two experiments is in the process of being evaluated.

KEYWORD DESCRIPTORS: Teratology; thioxanthone.

TITLE: "Evaluation of the Environmental Toxicant, Vinyl Chloride Monomer"

CONTRACTOR'S PROJECT DIRECTOR: C. C. Lee, Ph.D.

PROJECT OFFICER (NIEHS): J. S. Woods, Ph.D.

DATE CONTRACT INITIATED: December 31, 1974

CURRENT ANNUAL LEVEL: \$108,000

PROJECT DESCRIPTION

OBJECTIVES: This contract has been concerned with the toxic effects of the commercially available dithiocarbamate fungicides, ferbam and thiram, and their metabolic products.

More recently, experiments have been designed to further determine the laboratory parameters associated with the toxicity of vinyl chloride and other monomers. The specific objectives of this new study can be summarized as follows: 1) to determine the biochemical and pathological alterations in specific organ functions of rats and mice chronically exposed to atmospheres containing vinyl chloride monomer (VCM), 2) to establish and correlate response profiles of VCM for individual species with clinical data derived from epidemiological studies in humans and 3) to evaluate the utility of data derived from chronic toxicity studies of VCM in laboratory animals with regard to more effectively predicting toxicity in humans.

METHODS EMPLOYED: Conventional chromatographic procedures, U.V., visible, I.R. spectroscopy, histological techniques, radiotracer analyses, hematology, clinical blood chemistry, immunological tests, and cytogenic analyses.

MAJOR FINDINGS: With regard to the toxicology of dithiocarbamates, the studies of the metabolism and excretion of ferbam-³⁵S and ferbam-¹⁴C have continued. After oral administration of the appropriate fungicide, ³⁵S radioactivity was recovered in the exhaled air and in the urine; the ¹⁴C was excreted mainly in the urine and was not found in expired air. A small but significant amount of radioactivity was excreted in the milk and the bile, and some of the radioactivity was determined in the fetus. Metabolites were present in the urine, bile, milk and exhaled air. Cytotoxicity testing with ferbam, thiram and maneb suggested similar subcellular effects. Thiram did not produce a significant effect on fertility.

Embryotoxicity was demonstrated when pregnant rats were administered high doses of thiram, but significant malformations were not observed. Chronic toxicity studies of ferbam and thiram are being conducted and data should be available in the near future.

The vinyl chloride monomer studies will be initiated in June 1975.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: These investigations will establish the effect and nature of chronic toxicities of selected environmental chemicals using rats and mice.

This information will serve to form a rational basis for evaluation of the potential human health hazards produced by the use and exposure of these chemicals.

PROPOSED COURSE: To continue the specified objectives of the contract in relationship to the assessment of chronic toxic effects of various environmental chemicals. This contract also provides the NIEHS with a "quick response" capability to study environmental chemicals that are thought to be potentially hazardous to human health.

KEYWORD DESCRIPTORS: environmental toxicant; vinyl chloride monomer; biochemical and pathological alterations in specific organ functions; response profiles; epidemiological studies; chronic toxicity; "quick response" capability; environmental chemicals; human health.

MILES LABORATORY - ELKHART, INDIANA (NIEHS-73-2113)

TITLE: "Genetic Tests to Characterize Specific Locus Mutants in Neurospora crassa"

CONTRACTOR'S PROJECT DIRECTOR: L. T. Chang, Ph.D.

PROJECT OFFICER (NIEHS): F. J. de Serres, Ph.D.

DATE CONTRACT INITIATED: April 9, 1973

CURRENT ANNUAL LEVEL: \$54,425

PROJECT DESCRIPTION

OBJECTIVES: The primary objective of this contract is the genetic characterization of presumptive adenine-3 (ad-3) mutants of Neurospora crassa derived from experiments at the Institute from forward-mutation experiments with various environmental chemicals as well as various chemical carcinogens.

METHODS EMPLOYED: Stock cultures of presumptive ad-3 mutants have been characterized by a series of heterokaryon tests with various standard tester sets which determine the genotypes of the mutants, complementation pattern of ad-3B mutants showing allelic complementation and also distinguishes point mutations from chromosome deletions. During the latter part of FY 74, the contract was expanded to include making mutants homokaryotic and to prepare silica gel stock cultures.

MAJOR FINDINGS: During the past year, tests on mutants from a variety of experiments have been successfully completed. These include heterokaryon tests on mutants induced in haploid strains, predominantly gamma-ray induced mutants in six different UV-sensitive strains as well as the standard wild-type strain. In addition, mutants from a wide variety of experiments with various two-component heterokaryons were also completed. Mutagens used in treatment were: (1) various chemical carcinogens, (2) UV and (3) structural analogs of hycanthone and lucanthone.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: This contract relates to various programs in this Institute where it is essential to characterize the mutants induced at specific loci in this organism with regard to the type of lesion (e.g. point mutation or chromosomal deletion as well as the nature of the damage to the DNA at the molecular level). This is essential in our program to determine the correlation between carcinogenic and mutagenic activity as well as to determine whether chemical carcinogens characteristically produce any particular type of lesion at the molecular level. In addition, the characterization of the specific locus mutants produced by environmental chemicals enables us to better determine their relative hazard for man.

PROPOSED COURSE: During FY 76, the majority of the mutants analyzed will come from experiments with (1) carcinogen-induced mutants in excision-repair deficient strains, (2) carcinogen-induced mutants in standard wild-type strains, (3) radiation-induced mutants in two-component heterokaryons homozygous for excision-repair deficient strains (uvr-2 or upr-1) and (4) carcinogen-induced mutants in the latter strains as well as in heterokaryon 12.

KEYWORD DESCRIPTORS: Genetic characterization, mutants, Neurospora crassa, chemical carcinogens.

TITLE: "Birth Defects in Vietnam"

CONTRACTOR'S PROJECT DIRECTOR: Dr. Philip Ross

PROJECT OFFICER (NIEHS): Dr. John A. Moore

DATE CONTRACT INITIATED: September 15, 1974

CURRENT ANNUAL LEVEL: \$7,000

PROJECT DESCRIPTION

OBJECTIVE: To examine birth records of the Vietnamese for possible correlation with exposure to herbicides and birth defects.

METHODS EMPLOYED: Statistical studies of birth records in Vietnam.

MAJOR FINDINGS: The data currently is in the process of being analyzed to determine if there is a correlation between exposure to herbicides and the incidence of birth defects. Evaluation of the data should be completed early in Fiscal Year 1976.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: A wide variety of herbicides are used increasingly throughout the world. Therefore, it is important to attempt to understand the mechanisms of action of such compounds and to determine what, if any, effects such compounds produce.

PROPOSED COURSE: To analyze data and prepare a draft of the results. A final report is expected in mid-Fiscal Year 1976.

KEYWORD DESCRIPTORS: Exposure to herbicides; correlation with birth defects.

INFORMATION CENTER COMPLEX/INFORMATION DIVISION
OAK RIDGE NATIONAL LABORATORY - OAK RIDGE, TENNESSEE 37830
(Interagency Agreement Between NIEHS and ORNL)

TITLE: "Environmental Mutagen Information Center"

CONTRACTOR'S PROJECT DIRECTOR: Mr. John S. Wassom

PROJECT OFFICER (NIEHS): H. V. Malling, Ph.D.

COLLABORATING INSTITUTE: National Cancer Institute; Energy Research and
Development Administration

DATE CONTRACT INITIATED: July 1, 1970

CURRENT ANNUAL LEVEL: \$132,000

PROJECT DESCRIPTION

OBJECTIVES: The mission of the Environmental Mutagen Information Center (EMIC) is to collect, organize and disseminate chemical mutagenesis information.

MAJOR ACCOMPLISHMENTS: EMIC's data file at the end of March 1975 contained 75,500 bibliographic entries. Of these, approximately 14,000 have been indexed with respect to agent(s), organism(s), and Chemical Abstract Service Registry Number(s). The Center processes requests for information at a rate of 2-3 per day. These requests come primarily from governmental agencies in the United States or government supported research in universities. Several requests from other countries are also received.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Many compounds with widespread distribution in man's environment are known to be mutagenic in laboratory test organisms. Man's constant contact with mutagens may pose more danger to the genetic health of the human population than does radiation. The awareness of this problem among scientists, administrators and laymen created a need to make reference to published reports on tests for chemical mutagenesis readily available. This is important because these reports are scattered throughout a great number of publication sources (1550). EMIC has helped several national commissions in collecting and extracting the data from the literature. For the Institute, and especially the Environmental Mutagenesis Branch, EMIC saves more than one man-year of time for the personnel in the Branch by supplying their information needs.

PROPOSED COURSE: EMIC will continue to monitor the world scientific literature for reports on chemical mutagenesis. It will issue its annual literature survey in the middle of the fiscal year. This report will include an index to test organisms or test objects as well as indexes for agents and selected title keywords. As great a portion as possible of EMIC's total bibliographic entries will be keyworded with respect to agent, test organism and test object. EMIC will continue to answer selected requests for special information from the scientific, educational and industrial communities. Tabular

extracts of the data from literature will be added to the data file as they are prepared in conjunction with the timely state-of-art reports on subjects of current scientific interest.

KEYWORD DESCRIPTORS: Environmental Mutagen Information Center (EMIC), data file, agents, organisms, Chemical Abstract Service Registry number.

PUBLICATIONS

Brown, M. and EMIC Staff: The application of microbial assay systems as prescreens for detecting potential carcinogenic chemicals (in preparation for submission to Journal of the National Cancer Institute)

EMIC Staff: A survey of the 1971 literature of chemical mutagenesis, ORNL-EMIC-2, 1973.

EMIC Staff: A survey of the 1972 literature of chemical mutagenesis, ORNL-EMIC-3, 1974.

EMIC Staff: A survey of the 1973 literature of chemical mutagenesis, ORNL-EMIC-4, 1975.

Huff, J.E. and Wassom, J.S.: Health hazards from chemical impurities: Chlorinated dibenzodioxins and chlorinated dibenzofurans, Intern. J. Environ. Studies, 6: 13-17, 1974.

Malling, H.V. and Wassom, J.S.: The mutagenic action of environmental chemicals (in preparation for The Handbook of Teratology, to be published by Academic Press, New York, James Wilson and F. Clark Frazier, Editors).

Shelby, Michael D., de Serres, F.J., and Stine, Gerald J.: Ultraviolet inactivation of conidia from heterokaryons of Neurospora crassa containing UV-sensitive mutations, Mutation Research, 27: 45-58, 1975.

Shelby, Michael D., Stine, Gerald J., and de Serres, F.J.: Gamma-ray inactivation of conidia from heterokaryons of Neurospora crassa containing UV-sensitive mutations, Mutation Research (in press).

Von Halle, E.S., Generoso, W.M., and EMIC Staff: Chemical mutagenesis in mammals. A survey on the effects of chemicals on mammalian germ cells, ORNL-EMIC-5, 1973.

Wassom, John S. and Takenaka, Yusuke: Activities of the Environmental Mutagen Information Center, Review Journal of the National Institute of Hygienic Sciences, (Japan) Vol. 7(1): 1-17, 1974.

Wassom, John S.: The Environmental Mutagen Information Center (EMIC), Mutation Research, 21: 242-243, 1973.

TITLE: "The Interaction of Specific Air Pollutants with Lung Lipids"

CONTRACTOR'S PROJECT DIRECTOR: R. Bollinger, Ph.D.

PROJECT OFFICER (NIEHS): Phillip W. Albro, Ph.D.

DATE CONTRACT INITIATED: January 1, 1972

CURRENT ANNUAL LEVEL: \$167,757

PROJECT DESCRIPTION

OBJECTIVES: To evaluate the effects and mechanisms of interaction of specified gaseous compounds with lung membrane lipids in vivo and in vitro; to clarify the chemistry and biochemistry of such interactions; to attempt to correlate the structural changes observed on a chemical level with any significant functional changes; and to examine possible reversal, protective or preventative approaches.

METHODS EMPLOYED: The basic test animal is the rabbit; in vitro systems include lung slices, lung mitochondria and cells from lung washes. Various conventional chromatographic procedures, NMR and IR spectroscopy and histological techniques are used. ESR is used to detect alterations in membrane components in situ.

MAJOR FINDINGS: (1) In vitro exposure of rabbit lung to 1 ppm SO₂ resulted in a greatly reduced level of fatty acid β -oxidation and, apparently, a reduced level of activation of fatty acids for lipid synthesis. However, protective mechanisms appear to prevent this effect in vivo. (2) Various freons differ in their tendency to inhibit or activate lipase activity in the lung; those freons that activate lipases also appear to uncouple oxidative phosphorylation. (3) N₂O also appears to activate lipase in the lung, while NO₂ inhibits it.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: This project studies in detail a specialized aspect of the general chemical responsiveness of the lung to commonly inhaled substances, and thus should ultimately aid in predicting responsivity to other substances of interest.

PROPOSED COURSE: Termination (project complete).

KEYWORD DESCRIPTORS: Air pollutants; lung lipids; gaseous compounds; lung membrane lipids; reversal; rabbit; inhaled substances.

TITLE: "Collection and Storage of Biologic Specimens Exposed to Orally Ingested Asbestos Fibers"

CONTRACTOR'S PROJECT DIRECTOR: R. E. Carter, M.D.

PROJECT OFFICER (NIEHS): J. L. Braun, Ph.D.

DATE CONTRACT INITIATED: November 5, 1973

CURRENT ANNUAL LEVEL: \$43,000

PROJECT DESCRIPTION

OBJECTIVES: Collection and storage of specified biologic samples from human and non-human sources having varying exposures to oral ingestion of asbestos fibers; and, quantification of the asbestos fiber residue in selected samples.

METHODS EMPLOYED: Collected specimens will be identified and preserved to assure usefulness for current and future analyses. Quantification of asbestos fiber residue in human tissue specimens will be attempted using electron microscopy and electron diffraction and X-ray dispersive analytic techniques.

MAJOR FINDINGS: The contractor has been successful in collection and storage of specimens as proposed. Analysis of radiologic materials in process.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: This study will produce asbestos tissue burden information basic to determination of the health hazard potential of such exposure. Such studies are an integral part of the NIEHS efforts to determine the magnitude and significance of hazards inherent in long-term, low-level exposures to various environmental agents.

PROPOSED COURSE: The contract will be continued to provide for long-term retention of specimens collected under this contract.

KEY WORD DESCRIPTORS: Environment, health, epidemiology, asbestos

CANCER RESEARCH AND TREATMENT CENTER - THE UNIVERSITY OF NEW MEXICO
ALBUQUERQUE, NEW MEXICO (NO1-ES-5-2101)

TITLE: "Relationship Between Mortality and Ambient Air in the New York City Metropolitan Area"

CONTRACTOR'S PROJECT DIRECTOR: Robert W. Buechley, Ph.D.

PROJECT OFFICER (NIEHS): Michael D. Hogan, Ph.D.

DATE CONTRACT INITIATED: September 16, 1974

CURRENT ANNUAL LEVEL: \$42,229

PROJECT DESCRIPTION

OBJECTIVES: The major objectives of this research are: (1) To analyze daily mortality data from the New York metropolitan area from the period 1967-1971 (or later, if possible) using previously developed methodology to assess the possible correlation between mortality and air pollutant concentrations, particularly sulfur oxides; and (2) To perform analyses to ascertain comparability between this later determination and the determination previously made by the principal investigator for the five year period from 1962-1966.

MAJOR FINDINGS: Preliminary (and incomplete) analyses performed to date indicate that apparent correlations between daily SO₂ levels and daily fluctuations in the mortality ratio persist in spite of a marked reduction in the absolute levels of SO₂ in the study area during the latter time period.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: With the emergence of the "energy crisis" and the potential increased use of fuel sources high in sulfur content, the significance of an investigation of the possible association between fluctuations in daily mortality ratios and measures of sulfur oxide levels is clear.

PROPOSED COURSE: The project will be terminated this fiscal year, the preliminary findings will be verified, and an attempt will be made to interpret the meaning of any correlation (or lack of correlation) between mortality and sulfur oxide fluctuations.

KEYWORD DESCRIPTORS: SO₂, air pollution, daily mortality

University of North Carolina
Chapel Hill, N. C.
(NIH-75-C-337)

TITLE: "Determination of the Sites for the Accumulation of ^3H -Imipramine in Lung Tissue by the Use of Autoradiography"

CONTRACTOR'S PROJECT DIRECTOR: Walter Stumpf, M. D., Ph.D.

PROJECT OFFICER (NIEHS): T. E. Eling, Ph.D.

DATE CONTRACT INITIATED: February 1, 1975

CURRENT ANNUAL LEVEL: \$17,905

PROJECT DESCRIPTION

OBJECTIVES: To determine the locations of accumulated imipramine in lung tissue and to determine if accumulation of basic amines is associated with lung phospholipid.

METHODS EMPLOYED: The isolated perfused rabbit lung is used to study the accumulation of ^3H -imipramine in lung tissue. The isolated perfused lung containing ^3H -imipramine is examined in either frozen section or after fixation with gluteraldehyde at both light and electron microscopic levels.

MAJOR FINDINGS: Preliminary experiments are now in progress to determine the best methods for examining the lung tissue for autoradiography at the electron microscopic level.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Accumulation of basic amines in lung tissue has been associated with pulmonary hypertension and phospholipidosis. This contract attempts to provide important information needed to understand why basic amines accumulate in lung tissue and how basic amines induce lung phospholipidosis.

PROPOSED COURSE: After the best tissue preparation method is determined, the cellular location of ^3H -imipramine accumulated in the isolated perfused lung will be investigated by autoradiography.

UNIVERSITY OF WASHINGTON - SEATTLE, WASHINGTON
(NIH-NIEHS-74-2151)

TITLE: "Detection of Point Mutations in Somatic Cells"

CONTRACTOR'S PROJECT DIRECTOR: Peter E. Nute, Ph.D.

PROJECT OFFICER (NIEHS): Heinrich V. Malling, Ph.D.

DATE CONTRACT INITIATED: January 1, 1974

CURRENT ANNUAL LEVEL: \$75,000

PROJECT DESCRIPTION

OBJECTIVES: It is likely that an increase in the mutation rate in the human population would be detrimental, yet there is no system developed to monitor the human population for point mutations. The purpose of this investigation is to develop a simple system to measure point mutations in readily accessible human somatic cells. It is likely that the somatic cell mutation rate would reflect the germ cell mutation rate, and the rate of introduction of genetic defects into the human population.

METHODS EMPLOYED: Many human red cells contain small amounts (less than 1%) of the two types of fetal hemoglobin. The two types differ by one amino acid, and are called A_γ and G_γ . However, rare individuals have genetic disorders of hemoglobin production in which there is only one type of fetal hemoglobin produced. The same mutation is expected to occur in individual red blood cells within many humans, though very rarely. The research seeks to produce antibodies specific to each of the two types of fetal hemoglobin, using antigen blood from the rare individuals having only A_γ or G_γ . The two antibodies will be conjugated with different colored fluorescent dyes, and red blood cells from normal subjects will be exposed to the two antisera. Any cells containing only one fetal hemoglobin type will be identified by only one antibody and will fluoresce in only one color, while normal fetal hemoglobin containing red blood cells will fluoresce in both colors. Attempts have been made to produce antibodies specific for other hemoglobin variants.

MAJOR FINDINGS: Monospecific antibodies to variant human hemoglobins can be acquired and purified. Differential fluorescent staining of variant cells among normal cells has been achieved with fluorescent labeled antibody.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: A system able to detect deletions in the genome of readily accessible somatic cells would allow estimation of the human population rate. It would allow investigation of individuals and populations exposed to a mutagenic risk. There is no simple technique for screening at present. The antisera would also be useful in investigation of thalassemia and related genetic hemoglobin disorders.

PROPOSED COURSE: When the technique is available, it will be used to investigate somatic cell mutation rates with respect to age, and to investigate popu-

lations at mutagenic risk such as patients receiving cancer chemotherapy and those exposed to radiation. Screening programs would be able to identify populations exposed to mutagenic substances.

KEYWORD DESCRIPTORS: Mutation, somatic cell, human, red blood cell, fetal

TITLE: "Mutation Studies with Cultured Human Cells"

CONTRACTOR'S PROJECT DIRECTOR: Robert DeMars, Ph.D.

PROJECT OFFICER (NIEHS): F. J. de Serres, Ph.D.

DATE CONTRACT INITIATED: June 15, 1972

CURRENT ANNUAL LEVEL: \$86,059

PROJECT DESCRIPTION

OBJECTIVES: Develop a mutational assay system which will provide a means of monitoring mutations among somatic cells of humans. This system shall utilize the hypoxanthine-guanine phosphoribosyltransferase (HGPRT) locus or the azaguanine phosphoribosyltransferase (AGPRT) locus.

METHODS EMPLOYED: The contractor will use normal diploid human fibroblasts in culture and select for mutants using either 8-azaguanine or 2,6-diaminopurine respectively to recover resistant mutants at each locus.

MAJOR FINDINGS: The azaguanine-selection system has continued to show consistent rates of spontaneous mutation in fluctuation tests with cultures from different individuals or repeats from the same individual. The contractor has clearly demonstrated the dose-dependent sensitivity of the fibroblasts to the known mutagen MNNG. The post-treatment lag period needed for maximum detection of mutants has been better described than in earlier work. Other experiments with nitrosofluorene have provided dose-response curves for the mutagenic activity of this carcinogen. Controls for preferential recovery of pre-existing mutants clearly indicated that the mutant recoveries after mutagenic treatment are not due to recovery of pre-existing mutants. More than 100 independently induced and spontaneous mutant cell lines have been characterized in the course of these studies to ensure that the resistant colonies counted are hereditary variants of cells and not transient phenocopies of some sort.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: This contract relates to our attempts to develop assays to screen environmental chemicals for mutagenic activity (e.g., production of gene mutations). The development of generalized methodology will make it possible to study spontaneous and induced forward-mutation at the HGPRT locus in any individual in the population. Individuals of portions of the population at high risk can also be examined to determine whether the "spontaneous" frequency of HGPRT mutants is unusually high. Given cell lines can also be used to screen chemicals for mutagenic activity. Humans heterozygous at the AGPRT locus can usually be identified by pedigree analysis, and fibroblast cultures from these individuals can be used for analysis at either locus.

PROPOSED COURSE: The genetic analysis of azaguanine resistance and diamino-purine resistance and other stable phenotypic changes in somatic cells will require several years. In addition, experiments will be performed to attempt to detect the induction of azaguanine resistant mutations in human fibroblasts by hycanthone and two of its chemical relatives.

KEYWORD DESCRIPTORS: Mutagenesis, somatic cells, HGPRT, AGPRT.

RESEARCH PROGRAMS

OFFICE OF THE ASSOCIATE DIRECTOR
FOR EXTRAMURAL PROGRAMS
RESEARCH PROGRAMS

NATIONAL RESEARCH INSTITUTE

SUMMARY STATEMENT

OFFICE OF THE ASSOCIATE DIRECTOR FOR EXTRAMURAL PROGRAMS

RESEARCH GRANTS

During FY-1975, 213 individual research grant applications were reviewed, an increase of 26 percent over FY-1974. Awards were made for 120 noncompeting and 63 competing projects from 1975 appropriations and 21 competing projects from funds released from FY-1973 appropriations. Thus, regular research awards totaled 204, an increase of 19.3 percent, for the amount of \$12.9 million. In addition, 19 applications for Research Career Development Awards (RCDA's) were reviewed and 10 were awarded for a total of \$241 thousand. Since RCDA's are now considered to be research instead of training grants, the total individual research grants supported by the National Institute of Environmental Health Sciences was 214 for a sum of \$13.144 million.

TRAINING PROGRAMS

Seventeen institutional training grants awarded prior to passage of Public Law 93-348 were continued for a total of \$2.215 million. Phaseout of these is continuing in accord to the provisions of the new law. The number has declined approximately one-third from the 28 supported last year. Ten of these have applied for renewal under the new Institutional National Research Service Program (see below). Twenty-five individual fellowship awards were made (15 under the Weinberger Program and 10 under the new Individual National Research Service Program) for a total of \$360 thousand, and seven Institutional NRSA's were awarded.

NATIONAL RESEARCH SERVICE AWARDS

On July 12, 1974, Public Law 93-348 was enacted providing authority for NIH and ADAMHA to make National Research Service Awards (NRSA's) for predoctoral and postdoctoral training. This program supercedes all previous training programs but provides for orderly phaseout of existing programs. Thus, the NIH currently has three separate training programs--the "old" and the "Weinberger" programs, which are being phased out, and the NRSA's, which are being awarded for the first time from FY-1975 funds.

Guidelines for NRSA's were released on May 1, 1975. The new policies apply to all new and continuation awards (grants and fellowships) with start dates on or after May 1. Individual NRSA's may be made to individuals selected as a result of national competition. For the present, NIH is making awards to individuals only at the postdoctoral level, although predoctoral awards are not precluded. Institutional NRSA's may be made to domestic public or non-profit private institutions for support of awards to individuals selected by the training program director at the institution. Grants may be made also to Federal institutions which are eligible under Section 507 of the Public Health Service Act. Support for predoctoral and/or postdoctoral trainees may be requested.

The objective of the NIEHS Research Service Awards is to increase the number of career scientists with expertise in the assessment of environmental factors in health, assessment of risk-benefit in terms of socio-economic considerations, and in development of preventive and therapeutic health care programs consistent with competing human needs. Specific areas covered by the program are (1) environmental toxicology, (2) environmental pathology-pathophysiology, (3) environmental epidemiology, and (4) environmental biology. General provisions for the awards stipulate that they not be used to support studies leading to the M.D., D.O., D.D.S., D.V.M., or other similar degrees, or to support residencies. Research trainees in clinical areas are expected to devote their time to proposed research training and to confine clinical duties to those which are a part of the research training.

To be eligible, a trainee must be a citizen or a noncitizen national of the United States or have been lawfully admitted for permanent residence at the time of application for individual applicants or at the time of appointment in the case of an award through an institutional grant. Individuals on temporary or student visas are not eligible. Predoctoral trainees must have received a baccalaureate degree and must have been formally admitted by the sponsoring institution. An individual at the postdoctoral level must have received a Ph.D., M.D., D.O., D.D.S., D.V.M., O.D., D.P.M., Sc.D., D. Eng., D.N.S., or equivalent domestic or foreign degree. Awards may be made only in those areas where the need for research personnel has been determined by the Secretary, Department of Health, Education, and Welfare, and which are specified in the announcement by the National Institutes of Health.

Institutional grants may be made for project periods of up to five years, and may be renewable. No individual may receive more than three years of support in the aggregate, whether directly or through an institutional grant. Awards are normally made in 12-month increments with additional support dependent on satisfactory progress and availability of funds. No award may be made to an individual for less than nine months except with the prior approval of the awarding unit. All individuals are required to pursue research training on a full-time basis.

Each application will be evaluated by initial peer review groups and is subject to review and approval by the appropriate Council or Board of the NIH. Applications for individual awards will be evaluated on the basis of past academic and research records, the research training proposal, the sponsor's general qualifications, the training environment, research goals in terms of research training opportunities specified by NIH, publications, reference reports, and other relevant information. Institutional applications will be evaluated on the basis of records and qualifications of participating faculty, the proposed research training objectives and program design, previous training record of the program and its ability to attract high caliber students, institutional commitment, facilities and environment, and relationship of the proposed program goals to the need for the training in specified areas.

The financial conditions of awards are somewhat different from those of previous programs. For predoctorals, an annual stipend of \$3,900 is offered. For postdoctorals, the annual stipend is determined by the number of years of

relevant postdoctoral experience at the time of award. It ranges from \$10,000 to \$13,200 during the first year, with increases of \$400 per year during each subsequent year of training. There is no allowance for dependents. Upon request, the sponsoring institution will be provided up to \$3,000 per annum for each awardee. Upon completion of their training, recipients of awards are expected to engage in biomedical or behavioral research or teaching for a period equal to the period of support. However, in the event suitable research or teaching positions are not available (as determined by the Secretary, DHEW), there are several alternatives. These are specified in the guidelines and take into account the availability of appropriate jobs, the type of training of the individual and the needs in related areas of service.

A National Research Service Award may not be held concurrently with another Federally-sponsored fellowship or similar award which provides a stipend or otherwise duplicates provisions of the award. This provision does not apply to sabbatical salary. An individual may accept concurrent educational remuneration from the Veterans Administration (e.g., G.I. Bill) and loans from Federal funds.

UNIVERSITY-BASED CENTERS

University-based Centers are an integral part of the strategy of NIEHS in serving the national needs in environmental health research. They evolve as a consequence of the demonstrated ability of scientists within the same institution to perform high quality, multifaceted research in areas of high relevance to the mission of NIEHS and to attract and train outstanding students to meet future needs of the Nation. During FY-1975, the NIEHS supported seven Centers with funds totaling \$5.270 million.

Communication between the Centers and this Institute is maintained at an effective level through biannual meetings of the Center Directors, frequent visits and telephone contacts by Extramural Staff and through the National Advisory Environmental Health Sciences Council's Subcommittee for University-based Centers. The Subcommittee provides liaison between the Institute, the Advisory Council, and the Center Directors. It visits each Center in turn to discuss research problems and accomplishments, new environmental concerns, and program directions.

The tenth semiannual meeting of the Center Directors was held on May 19-20, 1974, at the New York University facility in Sterling Forest, New York, with Dr. Norton Nelson as host. Dr. Robert Williams, Technical Deputy on the Ford Foundation Study on the Energy Problem, discussed major issues related to the Study. The afternoon was devoted to presentation of program highlights by the Center Directors. It was felt that inadequate time was allowed for effective review of the programs. Consequently, it was agreed that more time would be made available at future meetings for this purpose. It was decided also that the Directors of the NIEHS-supported Program Projects should be invited to future meetings to present highlights of their programs.

The eleventh semiannual meeting was convened on October 6-8, 1974, at the Harvard University School of Public Health, with Dr. James Whittenberger as host. On Sunday evening, Dr. Alvin Weinberg, Director, Energy Research and

Development Office, discussed planning in the Federal Energy Administration. The following day there was a symposium on environmental health hazards to populations from development of various energy sources. The rest of the meeting was devoted to reports of activities in the individual Centers.

The University-based Center Subcommittee of the Council met prior to the Council meeting to discuss the history and functions of the Subcommittee as they evolved along with the Center program. The Center grants program concept, with emphasis on the advantages and disadvantages peculiar to these large integrated research and training mechanisms, was discussed with all members participating. The discussion is particularly important at present because the concept of large complex program grants is being questioned and there are increasing pressures to reduce or discontinue the funding of Center programs. Many who have intimate knowledge of the Center grant program and its concepts are convinced that a cutback in the program would be counter-productive. They contend that scientific productivity and cost/effectiveness experience would seem to make the program more attractive in times of tight money. There are weaknesses in the program to be sure, and one of the purposes of future discussions will be to identify and correct them.

SUPPORTED RESEARCH HIGHLIGHTS

During the year, grantees made substantive contributions to each of the categorical programs of the Institute. The etiological relations of bioactive environmental agents, some new and some with long-standing usage, in human health were illuminated further. Progress was made in understanding the basic toxicological and pathological events in pulmonary and cardiovascular diseases, reproductive and genetic anomalies, neurological disorders and various other syndromes ascribable to environmental factors. Parallel advances were made in analytical methodology for specific toxicants and noxious dusts and in development of quantitative test systems for carcinogenesis and mutagenesis. Highlights from a number of programs which illustrate these achievements are presented herewith.

RESEARCH HIGHLIGHTS

Etiology of Environmental Diseases and Disorders

In accord with the objectives of the Etiology Program, grant supported research projects have provided further information that serves to establish causal associations between environmental factors and disorders in man and/or representative animal models. Viewed in terms of the total program, there has been a notable progression of research activities from purely etiological considerations toward more fundamental, mechanistically oriented studies. This accounts for the fact that many of the projects reported in this Program Area last year are highlighted in one or more of the other Program Areas this year.

Pesticides

Organophosphates. Dr. Sheldon Murphy and his colleagues (Harvard University) have shown that there is a marked difference in susceptibility of mammalian, avian and piscine species to organophosphate insecticides. The relatively new compound ozinphosmethyl was found to be much more toxic in mice and fish than are parathion or methyl parathion. The relative toxicity appears to more closely related to the rate of recovery from AChE inhibition than to the rate of onset of AChE inhibition.

In the course of these studies, a gas chromatographic method was developed for analysis of parathion and methyl parathion. The GLC and the older anticholinesterase method give comparable results. For routine tests, the anticholinesterase method is more rapid than the GLC method because less sample extraction and clean-up time is required. However, the GLC method has the advantages of ease of standardization and of measuring the disappearance of thiophosphate substrates in the same incubation system used for metabolic determinations.

Dr. Dorothy Woolley (University of California) has studies in progress on the comparative effects of parathion on AChE in mice, rats, and monkeys. She has found that the rate of recovery of blood AChE activity is more rapid in mice than in monkeys following a single per os administration of parathion. The behavior of both mice and monkeys was affected by parathion at doses that did not produce other discernible symptoms of poisoning.

Paraquat. Dr. Eddie Wei (University of California) has attempted to clarify the site and mode of toxicity of the herbicide paraquat. Paraquat poisoning in man and experimental animals results in hyperplasia of bronchial epithelium and proliferation of alveolar cells, which suggests that the compound may be carcinogenic. In pulmonary adenoma tests with strain A mice as developed by Shimkin, which measures the predilection of chemicals to increase the formation of lung adenomas, paraquat did not increase the incidence or multiplicity of lung adenomas. This is in accord with previous long-term studies in rats and beagle dogs, which also indicated no carcinogenic activity of paraquat.

Hydrazines. Dr. Frank Dost (Oregon State University) has continued studies on the biological effects of hydrazines, which mimic diabetes in man. He has attempted to determine the median lethal dose in mice for several hydrazines but found that lethality is not a suitable parameter for determining potential or relative hazard of the compounds because the response is variable and the required dose is very high. He found several enzymes to be better and more useful indices of chronic exposure. Metabolism of methylamine and gamma aminobutyric acid was sharply inhibited. An exposure of 0.025 mole of hydrazine daily was equated with exposure to about 8 ppm hydrazine in the atmosphere, assuming no surface adsorption of the compound. Metabolism of ornithine was also inhibited in acute treatments. Minimum effective acute doses and the effects of chronic treatment are being determined.

Polychlorinated biphenyls (PCB's). Studies by Dr. Roy Kirkpatrick (Virginia Polytechnic Institute) show several types of interactions between the effects of PCBs and such parameters as diet, animal density and species. White mice, white-footed mice, and cottontail rabbits were compared for effects relative to variable PCB dose, diet and density. A significant PCB x diet interaction was observed on liver weights, sleep times and plasma corticoid levels. In white mice, PCB in the diet for two weeks increased liver weights, reduced sleep times and elevated plasma corticoid levels. Conversely, PCB significantly reduced plasma corticoid levels in white-footed mice. Liver weight and sleep time effects were similar to those in white mice. In cottontail rabbits, Aroclor 1254 in the diet for 12 weeks increased liver weights and significantly reduced sleep times but had no effect on plasma corticoid levels or on male or female reproduction as measured by sperm counts, number of corpora lutea and number of embryos. These findings point up the need for further comparative physiological studies on PCBs and other agents.

Air Pollutants

Automobile exhausts. Studies begun several years ago by Dr. Frank E. Speizer (Harvard University) were continued. The studies were designed to follow prospectively a relatively healthy segment of a population of policemen over an extended period of time with respect to development of chronic nonspecific respiratory disease, hypertension and other cardiovascular diseases. Intense exposure to automobile exhaust and cigarette smoking were the major environmental factors under study.

Although automobile exhaust as a respiratory pollutant may be significant, thus far its potential as a chronic low level irritant and its effects on pulmonary function over a four year time span in terms of the development of obstructive disease appears to be minimal. This is in contrast to studies in firefighters in parallel studies, which show a significant decline in pulmonary function over the course of only two years.

In connection with these studies, personal sampling techniques and devices have been developed which afford a flexibility in monitoring that was not previously available. The suitcase sampler can be transported easily and set up in the field to monitor exposure to fossil fuel pollutants of all kinds. The more personal sampling devices for CO, NO₂, Pb, and mass respirable dusts, besides being used in this study, are being used in a

study of firefighters to monitor exposure at the site of fires and appear suitable for use in other situations involving the gaseous and particulate by-products of combustion.

Pentachlorophenol. Previous studies by Dr. Ted Norton (University of Hawaii) have demonstrated the toxicity of pentachlorophenol and the levels of the compound in the bloodstream of wood treatment workers. The work has been extended to a consideration of exposure route, i.e., intraperitoneal injection vs inhalation, in order to clarify various aspects of disposition of the compound in the body. A new aerosol generator was designed which allows production of wide range of aerosol exposures. Using rats as the test organism, it was found that considerably higher tissue levels are reached when inhalation rather than i.p. injection is the primary route of exposure. Inhalation exposure also results in an increase in urinary excretion of the administered dose. This is attributed to a reduction in metabolism of the compound as compared to that administered i.p. since in the latter case, more of the compound passes through the liver before distribution to the rest of the body. This appears to be borne out by studies showing that the LD₅₀ in rats is 5-7 mg/kg upon inhalation, 30 mg/kg i.p. and 200 mg/kg os. This is another of an increasing number of examples demonstrating the critical importance of the route of exposure in the ultimate toxicity and hazard of environmental agents.

In other studies on pentachlorophenol metabolism in rats and humans, Dr. Norton has found only one metabolite, tetrachlorohydroquinone, in the urine. These studies are not considered conclusive with respect to the spectrum of metabolites that might be formed.

SO₂ and sulfuric acid mist. Dr. Mary Amdur (Harvard University) has continued work on irritant responses to inhaled SO₂ and aerosols. Current results indicate that the effects of SO₂ and 1 nM sulfuric acid droplets inhaled together are simply additive at low concentrations.

Further studies on the potentiation of irritant responses to SO₂ by aerosols of soluble ferrous and vanadium salts substantiate previous conclusions that the potentiation is due to catalytic conversion of SO₂ by the metal salts partially to sulfuric acid. These studies emphasize the importance of SO₂-particulate interactions in SO₂ effects and alerts us to the potential importance of such interactions in other types of inhaled gases and particulates.

Dr. Amdur has also studied the physical and chemical interaction of SO₂ with various oils. The rationale is that oil mists are important components of the atmosphere where SO₂ is most prevalent. SO₂ is soluble in medicinal grade mineral oil but shows no reactivity. In ARCO mineral oil it is both soluble and chemically reactive. This is true also of clean and used motor oil. The reactivity is greater with clean than with used motor oils but the solubility is essentially equal in both. Plans are being made to examine the response of animals to oils alone and in combination with SO₂ and to oils previously reacted with SO₂.

Asbestos. Dr. William Nicholson (Mount Sinai School of Medicine) has continued studies to document the extent, intensity and persistence of airborne asbestos pollution. Extensive data have been collected on the burden of amosite fibers in the lungs of occupationally exposed workers in asbestos insulation plants in Paterson, New Jersey; Tyler, Texas; and Port Allegany, Pennsylvania. Correlations are being made between the lung sites and burdens of fibers and fibrils and lung cancer.

Other studies involve lower levels of exposure and include analysis of dust samples taken from typical homes at various distances from factories as well as the analysis of lung tissues from individuals who lived in such homes before death. The dust samples typically contain amosite levels of the order of 10^{-4} to 10^{-5} grams per gram of dust. The levels in lung tissue specimens were found to be so low that quantitative data are difficult to obtain by the thin section technique. Thus alternative techniques are also being applied. These involve use of the optical microscope to determine the number of asbestos bodies in the tissues of exposed and controlled populations. Asbestos bodies form around amosite fibers in the lungs and may provide a useful quantitative index of exposure. Another alternative procedure is the digestion of large amounts of tissue and concentration of the residue. These techniques are in the developmental stage with the expectation of applying them in future quantitative studies when needed.

In June 1973, the entire facilities of the Environmental Sciences Laboratory under Dr. Nicholson were directed toward the quantitation of asbestos-like fibers in the drinking water of Duluth, Minnesota, and other cities which draw their drinking water from Lake Superior. In addition, the air of Duluth and Silver Bay and tissue of deceased long-time residents of Duluth were examined. Approximately 30 air and 30 tissue samples were analyzed and the results were presented to the Federal Court in Minneapolis, which was hearing a suit brought by the EPA, three states and several environmental groups to abate the source of pollution. Though the court ordered cessation of dumping of mining wastes into Lake Superior, the ruling was later reversed because the data on adverse health effects were considered insufficient and inconclusive. The problem is still under study.

Plumorary Defenses

Dr. Paul Southern (University of Texas, Southwest Medical School) has continued studies on the effects of NO_2 , CO and lead oxides on the ability of young male rats to handle aerosols of Streptococcus pneumoniae, Pseudomonas aeruginosa and Hemophilus influenzae. The studies include the in vivo pulmonary bactericidal capacity of alveolar macrophages and nonviable particle clearance via the mucociliary escalator. Limited results to date suggest that exposure of rats for three weeks to low levels of NO_2 substantially reduces defenses against P. aeruginosa. The effect appears to be on the mucociliary escalator, although long-term effects on macrophages have not been ruled out.

Extensive studies of pollutant effects on various aspects of alveolar macrophage activities and genesis are being conducted by Dr. Joseph Brain at Harvard University. Specifically, the studies deal with the sources of

alveolar macrophages--whether of pulmonary or hematopoietic origin--under varying conditions, mediators of macrophage response--whether hormonal or nervous--and alterations in the macrophage pool following inhalation or intratracheal injection of particles. Studies on the pathways of clearance of iron oxide aerosols in mouse lungs have clarified an old controversy as to the relative importance of airway vs lymphatic clearance pathways. The airway clearance route predominates in the early phases after exposure but there is eventually an increasing contribution of the lymphatic system. Factors influencing aerosol deposition in dogs, rodents and guinea pigs were examined using a technitium-⁹⁹ labeled aerosol. It was found that deposition in all species was greater at the top (anterior) than at the bottom (posterior) of the lung. This is in marked contrast to the pattern of effective ventilation.

Studies were begun also on the influence of species differences on aerosol deposition patterns. Latex casts were made of the entire respiratory tracts of mice, rats, hamsters, guinea pigs and rabbits to define the shapes and dimensions of the airways. Normal breathing patterns for these species were also monitored. It was found that with a number of idealizations, deposition is proportional to $W^{-2/3}$, where W is the total body weight of the animal. This theory agrees with experimental data well enough to encourage further research aimed at discovering the scaling laws by which results from deposition experiments in small test animals may be translated to man.

Somewhat similar experiments on deposition of polonium-210 on hematite particles were carried out by Dr. John Little and his colleagues (Harvard University). Quite different patterns of ²¹⁰Po deposition and disposition were observed following administration of Po alone in saline and Po on hematite particles. ²¹⁰Po administered alone in saline was distributed throughout the lung. However, alveolar epithelial cells received two to three times the overall radiation dose of epithelial cells in the bronchi or bronchioles. After several months, essentially all of the activity remaining in the lungs was in the alveolar epithelium. Within the alveolar epithelium, a major portion of the activity was in Type I cells and at the end of eight months only these cells retained radioactivity. In the bronchial and bronchiolar epithelium, most of the activity was in ciliated and Clara cells. Occasional tracts occurs in basal cells but none or negligible amounts was found in goblet cells, tracheal epithelium or the lymphatic system.

Administered on hematite particles, the distribution of ²¹⁰Po was distinctly nonhomogeneous. The Po remained almost entirely with the hematite particles, and at any time beyond the first few hours, most of the activity was within macrophages. Within the same lung section ²¹⁰Po-hematite appeared in high concentrations in some areas and was entirely absent in others. Initially, the hematite was largely cleared up the mucous sheet but later was contained within macrophages.

The ²¹⁰Po retained in the lung on particles was almost entirely within interstitial macrophages surrounding alveolar ducts. The radiation dose was, therefore, not only very nonhomogenous but highly concentrated with respect to cells in this region. These studies relate directly to the etiology of

cancer induced by ^{210}Po . Further studies relating to carcinogenesis are reported in the subsequent section on Pathogenesis.

Heavy Metals

Lead. Dr. Louis Kopito (Children's Hospital Medical Center, Boston) has conducted studies on the role of lead in iron deficiency anemias and the susceptibility of anemic children to Pb insult. It was found that experimental rabbits developed mild to severe iron deficiency anemias about three to six weeks after treatment with lead acetate (5 mg every second day).

The red blood cell saturation capacity with Pb and Fe was shown to be dependent on previous exposure to Pb and Fe in approximately equal wt./vol. ratios. The prior presence of excesses of either element inhibited the penetration of the other into the RBC. This suggests that Fe and Pb may compete for the same binding sites. Similar experiments with Cd, Hg, Cu, Mn, Mg, Ca, Zn, Co, Ni, and Cr showed that none of these elements inhibited penetration of Pb into RBC. Thus, it appears that inhibition of Pb penetration is unique to Fe, which opens a new dimension in the study and understanding of mild chronic lead intoxication and points up the importance of mineral nutrition in the prevention of some types of metal intoxications.

Dr. Chester Wilpizeski (Thomas Jefferson University Medical College) has continued studies on the ototoxic effects of lead and mercury in monkeys. He found that the ototoxicity of lead is not very great even at levels so high as to cause debilitation and death. Lead intake for periods up to six months resulted in an average shift of about 10dB in the auditory detection threshold for pure tones. In comparisons between hearing losses measured by different procedures, it was found that the averaged evoked response (AER) measurements of brain cortex (dural electrodes) reflected more loss of auditory sensitivity than did the behavioral measurements. Though the full significance of this is not yet known, it might suggest that the so-called EEG audiometry used to assess infant hearing is invalid as a measure of perceptual processes in subjects with CNS impairment of a general nature. Further work on infant monkeys is planned to clarify this.

In work on ototoxicity of methyl mercury, Dr. Wilpizeski indicates that his findings do not permit conclusions made by Falk, Klein and Sanders of the National Institute of Environmental Health Sciences, published in Environmental Health Perspectives. Falk et al. concluded that anatomical damage was confined to opical (low audio frequency specific) receptor cells, and that the sensori-neural deafness reported in some mercury poisoned humans is not the result of CNS pathology. Dr. Wilpizeski's findings reveal severe changes in auditory processes, and the hearing losses extend well into the middle (4,000 Hz) and high tone (16,000 Hz) regions of the audio spectrum, with some threshold shifts greater than 85dB. He finds that induced nystagmus in the monkey is markedly curtailed or even eliminated after four to six weeks of intoxication and suggests that evaluation of ototoxic agents is inadequate if auditory and vestibular function is ignored.

The significance of these studies, if confirmed, is that the adult otoneural system is only mildly affected by chronic increased body burden of lead but

is damaged to a great extent by methyl mercury poisoning. Dr. Wilpizeski suggests that the purportedly poor hearing of older lead industry workers is not a consequence of lead exposure but of noise trauma and aging processes.

Metallic Mercury Vapor. Dr. Thomas Clarkson and his colleagues (University of Rochester) has continued work on metallic mercury vapor. Discussion continues on acceptable safe limits to occupational exposures with standards differing by a factor of ten from one country to another. Non-occupational exposure may become significant from the public health point of view for populations living close to points of release of the vapor. For example, air levels of mercury have been reported to exceed occupation safety limits in the village of Almaden, Spain--the site of the world's largest and most active mercury mining operation. High mercury levels have also been reported in the U.S.A. and Japan close to the point of emissions from mining operations, refineries and in fields where mercury fungicides are used. The primary objective of this program is to bring to bear new information on the biological properties of inhaled mercury vapor that will allow a more rational approach to the setting of industrial and ambient environmental air safety standards for mercury vapor.

In studies of five volunteers, quantitative information has been obtained for the first time on the degree of retention of inhaled mercury vapor in man. Following almost complete retention of vapor in the lung, it is excreted from the body slowly with a biological half-time of about 58 days. This indicates that workers occupationally exposed to the vapor will continue to accumulate the metal for about one year. From the practical point of view, blood and urinary mercury levels would not be a useful index of exposure until the worker had been employed for one year.

Studies on volunteers have identified a new pathway of excretion of mercury in man. Significant amounts (about seven percent of the inhaled dose) were excreted from the lungs as volatile mercury. This confirms previous work at Rochester on experimental animals and may explain the findings of Bramen in Florida of elevated mercury air levels in crowded rooms. The data suggest that measurement of exhaled mercury could be used as an index of recent exposure to mercury.

Studies on experimental animals and on volunteers may explain the phenomenon of tolerance to mercury vapor reported by Goldwater and his colleagues in the 1950's. They noted that some workers after long term exposure to the metal having apparently high body burden, nevertheless, showed no signs of ill health. Present studies have revealed that repeated exposure of animals to metallic vapor leads to the induction of a special metal binding protein, metallothionein. This protein is known to form tight complexes with metals and to protect tissues from the toxic effects of heavy metals, including cadmium and inorganic mercury. The studies show that levels of this protein in kidney tissue are dramatically increased by exposure to mercury vapor and mercury becomes bound to this protein. Furthermore, preliminary evidence from the volunteer studies indicates that mercury becomes bound to this protein in man after exposure to the vapor.

It was found that interactions may occur between mercury and other chemicals to which man is exposed, thus complicating assessment of hazard. Previous

studies have established that moderate doses of alcohol can affect the retention and excretion of mercury vapor. Studies on factors affecting the toxicity of inhaled vapor have now demonstrated that alcohol can protect animals against the toxic action of inhaled vapor. Alcohol may be one of many chemicals that act by inhibiting the conversion of elemental (Hg^0) to its toxic form, ionic mercury (Hg^{++}). The herbicide, aminotriazole, also acts in this way.

Foods

Coffee. The relationship between coffee drinking and cancer of the lower urinary tract (LUT) was investigated further by a case-control study of white women identified from hospitals in urban areas of Massachusetts and Rhode Island (Dr. Whittenberg et al., Harvard University). For women who usually drank more than one cup of coffee per day, the risk ratio of having LUT cancer was 2.1 compared to a risk of 1.0 for women who drank less or not at all. However, no dose-response relationship was demonstrated and there was no difference in the association of coffee with disease with regard to regular and decaffeinated coffee or perk and instant coffee. Use of creamers, saccharin, cyclamates or strongly brewed coffee also did not increase the risk of disease. The risk of smokers relative to nonsmokers was 1.6, which was a significant increase. However, the difference between this figure and 2.1 for coffee drinkers is questionable, leaving some question of the significance of coffee drinking in LUT cancer.

Artificial Sweeteners. The work of Golberg et al. (Albany Medical College) on the possible carcinogenesis of cyclamates and saccharin does not appear to support previous reports of carcinogenicity of these substances. Experiments have been conducted on monkeys given 200 mg cyclamate/kg/day for 322 weeks. These animals show no overt signs of toxic reaction. Body weights, hematological and standard biochemical parameters examined at frequent intervals have been within normal limits. Tests on saccharin at up to 500 mg/kg/day have been in progress for 252 weeks. Although one animal has died in each test group, in no case was death judged attributable to the treatment. Whether the monkey is different from other species tested for cyclamate and saccharin carcinogenesis remains to be resolved, but if the monkey is more representative of humans, the cancer risk to humans using artificial sweeteners does not appear to be real, at least within the time frame of these experiments.

Monosodium glutamate. There have been conflicting reports about the toxic effects of monosodium glutamate (MSG) in the hypothalamus of newborn mice, rats and monkeys. While most investigators agree that hypothalamic lesions are formed in mice, no such unanimity of views exists regarding neonatal monkeys. A detailed investigation by Golberg et al. by light and electromicroscopy has not revealed any hypothalamic lesions in neonatal monkeys given MSG at several levels over different periods of time. Other parameters studied include the maturity of the blood-brain barrier, the mode of entry of glutamate into the brain, and the manner in which this amino acid is handled by glial cells as well as changes in electrolyte composition of the cerebrospinal fluid.

Work on this project is nearing completion and conclusions drawn from the work are: (1) that the arcuate nucleus of the neonatal monkey is not susceptible to high doses of MSG, (2) the effects of MSG in rats are strain-specific, and (3) a lesion noted in the area of the median eminence of the neonatal guinea pig was apparently due to subcutaneous administration. Whether the same result would have been obtained by oral administration remains open to question.

If present indications regarding coffee-drinking, sweeteners, and MSG are confirmed, it appears that the lengthy and expensive furor about these substances will have been without real foundation and should serve as a warning against over-zealous and hasty incrimination of useful consumer (and industrial) products.

Natural Products

Ochratoxin A. Dr. Herman Meisner (Duquesne University) has succeeded in purifying and crystallizing ochratoxin A from *Aspergillus ochraceus*. Work on the mode of toxicity using the purified compound shows that the compound inhibits ATP synthesis and state III respiration but accelerates state IV respiration, which apparently is due to induced passive swelling of mitochondria.

Aflatoxins. Dr. Russell Sinnhuber et al. (Oregon State University) have continued studies on carcinogenicity of aflatoxin B₁ metabolites, using rainbow trout as the test model. Aflatoxin M₁, which occurs in milk, urine and tissues of animals fed B₁, was found to be a potent liver carcinogen but somewhat less active than B₁. Trout receiving as little as 20 ppb M₁ for a period of only five to thirty days developed hepatomas in twelve months.

Suggestive but not conclusive evidence was obtained that urine of humans known to have ingested peanut butter and corn contaminated with aflatoxin may contain carcinogenic aflatoxin metabolites. Extracts of the urine were incorporated into a test ration and fed to rainbow trout for thirty days. The fish were subsequently held on a control diet. After eleven months, there was a 28 and 15 percent incidence of hepatoma in trout fed urine from peanut butter and corn ingesters, respectively. The carcinogen does not appear to be aflatoxin B₁ or M₁ and remains to be identified.

Experiments were also conducted with aflatoxin Q₁, a product of the action of microsomes from the monkey on B₁. The product appears to be carcinogenic but dose-response relations have not yet been determined.

These investigators have also found potentially important differences in carcinogenicity of B₁ related to type of protein in the diet. Fish protein concentrate substituted for casein in the diet at identical levels gives a dramatically lower incidence of hepatoma and tumor growth rate. Body growth is the same on both diets. The essential difference between the proteins is lower proline and glutamic acid in the fish protein concentrate. It may well be that substances other than protein in the fish concentrate may be

responsible for antagonism of B₁ carcinogenicity. Nevertheless, the study points up the possible importance of diet in carcinogenicity of aflatoxins (and perhaps other carcinogens with similar modes of action).

Continued studies by this group of investigators on cyclopropenes (sterculate) as cocarcinogens have provided indications that these compounds may in fact be weak primary carcinogens in rainbow trout. Dietary levels of 50 ppm for as little as three days were sufficient to produce a six-fold increase in hepatoma incidence when 4 ppb B₁ was fed simultaneously.

Synthesis of a number of cyclopropene analogs has been achieved for studies on structure-activity relationships in cocarcinogenesis. These include dipropyl, dipentyl, dihexyl and dioctylcyclopropene and methyl malvalate and sterculate.

Further studies were conducted on induction of tumors with B₁ by exposure of trout eggs (embryos). Eggs were exposed for 15, 30 and 60 minutes to 0.5 ppm B₁. The eggs were then allowed to hatch and the fish were fed control diet containing 50 ppm of cyclopropene fatty acid. After 12 months, the tumor incidence was 41, 75 and 89 percent, respectively, with less than 3 percent in the controls. Because of the short exposure time required, this procedure has promise as a sensitive new test system for carcinogens.

Dr. Wallace Hayes (University of Alabama) has determined the LD₅₀ for a number of common mycotoxins for the rat and has tested combinations. He found the relative mortality to be in the order rubratoxin A > ochratoxin A > aflatoxin B₁ > citrinin. However, these are mortality data and the order does not necessarily hold for carcinogenesis. Combinations of aflatoxin-rubratoxin and ochratoxin-rubratoxin were more lethal than the individual mycotoxins at comparable total doses. LD₅₀ values ranged from 1.36 mg/kg for rubratoxin B to 18.2 mg/kg for citrinin. Pathological observations for the various compounds and treatments are incomplete.

Studies by Dr. William Carlton (Purdue University) on carcinogenic activities of toxigenic fungi have attempted to broaden understanding of species differences in responses. It was confirmed that Penicillium viridicatum toxins and sterigmatocystin in the diet can increase the incidence of pulmonary neoplasms in mice. Ochratoxin A does not appear to be carcinogenic in the mouse or rat. However, it is a potent nephrotoxin in the pig and especially so in the dog. These data combined with emerging observations of grain contamination indicate that ochratoxin A might be a significant environmental contaminant. The roles that ochratoxin A and other mycotoxins play in human renal disease are not known but epidemiological evidence suggests that fungal toxins may be significant in the development of endemic nephropathy in humans in the Balkans. It seems established that ochratoxin A is a cause of endemic porcine nephropathy in Denmark.

In a previous progress report, Dr. Benjamin Wilson (Vanderbilt University) described the principal hepatotoxins and pulmonary toxins produced by mold-damaged sweet potatoes and their structures. He has now determined the structure of a new hepatotoxic metabolite, 7-hydroxymyoporone.

Further studies on the mechanism of lung toxicity of 4-impomeanol have shown that approximately 50 percent of the i.p., nonlethal dose of the compound is excreted in the urine within two hours. Of the residual quantity retained by the body, the greatest concentrations occur in the lungs where binding is prompt and persists for at least 100 hours. Binding is covalent with at least 80 percent attached to lung protein and lesser quantities to nucleic acids. The extent of covalent binding to lung proteins correlated well with the degree of pulmonary edema and lethality.

These workers are continuing studies on the metabolite of Fusarium moniliforme responsible for the brain lesions seen in leukoencephalomalacia of equines. A newly discovered compound from this organism called moniloformin is being synthesized in order to provide sufficient quantities for equine feeding studies. It should be noted that the syndrome produced by this organism in equines is not produced in rodents or other species examined thus far. Thus, the significance with respect to man remains to be shown.

Physical Factors

Laser radiation. Dr. Karl Weiss (Northeastern University) has continued work on the effects of laser radiation on biological systems and photosensitive biomolecules, particularly flavins and visual pigments. By use of flash photolysis techniques he has found that photolysis of the retinyl chromophore in rhodopsin generates the triplet state. The triplet quantum yield for a model Schiff base structure such as that formed is essentially zero. This makes triplet state participation in the primary photochemical processes of vision unlikely. Further studies on other photolysis intermediates such as prelumirhodopsin are being pursued in efforts to elucidate the photochemistry of vision and other light responsive physiological processes.

Light. Dr. Frederick Urbach and his colleagues (University of Pennsylvania) have continued work on several aspects of the biologic effects of light. Concern has grown recently about possible alteration of the earth's atmosphere with resultant changes in the quality of light reaching the earth's surface. In addition, there is concern about the plethora of photoactive chemicals to which the population as whole is being exposed.

New equipment for monitoring, metering and measuring irradiation of experimental animals and man has been developed. This provides heretofore unavailable capabilities for diagnosis of light induced diseases of man, for treatment of certain diseases and for quantitative and qualitative studies of various spectra on tumorigenesis and other photobiologic phenomena.

Skin cancer can now be induced reliably in animals by use of the new equipment and techniques. The techniques also provide predictive capability with respect to the consequences of diminishing atmospheric ozone and the interaction of light and chemicals in photocarcinogenesis of the skin. For example, it was discovered that delayed hypersensitivity plays a protective role in visible light and UV-induced skin cancer. This may be of great significance because it suggests that stimulation of the immune system or other protective mechanisms can prevent or reduce the incidence of skin cancer and that immunotherapy might be of real value.

The complicated photochemical transformation of environmental carcinogens is being studied. It was found that different photoproducts are produced by light at different wavelengths. Some of the products are highly carcinogenic, others noncarcinogenic. The chemistry of the products is being investigated.

The problem of side effects in the phototherapy of hyperbilirubinemia in infants is being investigated. New spectrally narrow, highly active lamps have reduced treatment time and increased the rate of bilirubin destruction. However, serious side effects are possible, such as damage to unprotected retinas (particularly in the presence of oxygen), reduction of riboflavin, and changes in biological rhythms. These are being investigated to find ways to avoid unwanted consequences.

Dr. Leonard Harber (Columbia University) has continued studies designed to evaluate environmental agents for photosensitivity reactions in man and to determine the mechanisms of photosensitivity reactions in laboratory animals. The studies thus far indicate that porphyrins act as endogenous photosensitizers in the presence of porphyrin inhibitors and stimulators in plasma.

The search for a rapid test for diagnosis of erythropoietic photoporphyria has led to a useful impregnated filter paper test that is now in use.

Photoprotective studies have continued on the use of beta-carotene as an oral agent in the treatment of erythropoietic photoporphyria. Although still in the investigational stage, this compound appears to be an excellent therapeutic agent and is approaching international acceptance. Tests showed the compound to be effective in 49 of 53 patients studies. Efforts are being made to find other non-toxic systemic agents that will protect against 300 and 360 nm radiation.

Thermal effects. Dr. Pharo Gagge and his colleagues (John B. Pierce Foundation) have continued research toward understanding of man's physiological response to temperature and other factors in his surroundings. The purpose of the studies is to determine the factors that contribute to man's efficiency and comfort. Once these are verified it will be possible to correct suboptimal surroundings and to design buildings for optimal working efficiency in accordance with the nature of the work. A series of simplifying concepts have been used to describe man's heat exchange with his thermal environment.

Test Methodology Development

Analysis of CO. Dr. Jack Lambert (Kansas State University) has developed a fast convenient method for blood carbon monoxide that minimizes manual manipulation and is adaptable to automation.

Particle retention. Dr. Morton Lippmann (New York University Medical Center) has developed a new collimated scintillation detector for measurement of particle retention in the lungs. The new design, consisting of three cylindrically collimated one-inch diameter gamma-scintillation detectors arranged as an equilateral triangle, was constructed to replace the single

three-inch diameter stomach detector used previously. The particle distribution seen by the three detectors determines the mean position as well as the total quantity of activity within the stomach, permitting an accurate correction of the lung field measurements for counts coming from the stomach. Dr. Lippmann has also developed a spinning disc generator to produce aerosols with particle diameters less than 1 nm.

Test mouse populations. Dr. Edward Palmes (New York University Medical Center) has continued a project in progress for several years to determine the feasibility of using populations of mice as test subjects for measuring chronic effects of low-level exposure to environmental chemicals. Such populations have the potential for demonstrating effects of pollutants on animals in utero through old age as well as affording a means of measuring effects of combined social and chemical stresses on subsequent generations. Details of animal husbandry and record keeping have been developed and the data stored on tapes. Programs were developed for characterizing and comparing the populations in terms of factors such as life expectancy, reproductive indices and weight changes. It is felt that this procedure simulates human exposure to environmental chemicals much more closely than do the usual long-term feeding experiments.

Air pollution generating and monitoring system. Dr. Phyllis Hartroft and her colleagues (Washington University, St. Louis) have developed a programmed monitoring system with feed-back control to create preselected levels of gaseous and particulate mixtures typical of urban air pollution. An important improvement is the use of dry methods for gas monitoring as opposed to wet colorimetric methods previously employed. The technically difficult task of particulate generation is being solved by use of a dust feed mechanism with an optical sensor for each room in which experimental animals are kept.

In addition, work during the current year has led to development of a more suitable model for emphysema. A single intratracheal injection of elastase in hamsters gives rise to pulmonary lesions indistinguishable from the well-known papain-induced emphysema. Mortality from the lesion is low (10-20 percent). This model is useful for morphometric procedures relative to emphysemic conditions, including measurements of lung volume, mean linear intercept and internal alveolar surface area.

Pharmacology - Toxicology

The environmental Pharmacology and Toxicology Program seeks to develop understanding of the pharmacological principles that govern the site and severity of damage by environmental agents. Specifically, it deals with (1) the processes that determine the load and site of action of toxicants with which the body must cope (deposition, absorption, and transfer within the body); (2) the disposition of foreign substances (storage, toxication-detoxication, and excretion); (3) the functions and mechanisms of mediating systems (metabolism and enzyme induction, repression and inhibition); and (4) toxicity modifying factors (age, sex, species, nutrition, current and antecedent diseases, immunology, and genetics). Understanding these processes and their differences among individuals and species is fundamental to interpretation of time-dose-response relationships in all disorders caused by environmental factors.

A number of projects previously reported in the Etiology section have progressed to the point where the main focus is on further understanding of pharmacological and toxicological details. These projects have been moved to this section for this report.

Pesticides

Multifaceted studies. Dr. John Casida and his colleagues (University of California, Davis) have continued multifaceted studies on a wide range of agricultural and other chemicals, including biological control agents. In view of the scope of the work, it is feasible to indicate here only the areas and nature of the work rather than specific details.

The studies have importantly contributed to a better understanding of (1) the metabolism and biochemical behavior of organophosphates, carbamates, rotenone, pyrethroids, methylenedioxyphenyl synergists, thiocyanates, organotins, certain respiratory uncouplers and tetrachlorodibenzo-p-dioxins; (2) the photoalteration of rotenone, certain carbamates, pyrethroids, chlorinated methano-bridged cyclodienes and respiratory uncouplers; (3) the binding sites of rotenone, piericidin A and coenzyme Q in the respiratory chain components; (4) the biochemical bases for lesions leading to teratogenesis induced by certain organophosphorus and methylcarbamate compounds; (5) the toxicity, activity and mode of action of certain pesticide chemicals that uncouple oxidative phosphorylation; (6) biochemical factors involved in the development of resistance and in the genetics of resistance; (7) the importance of sensitizers in the photoalteration of pesticide chemicals; (8) the fate and mode of action of juvenile hormone analogs; (9) the factors that control the selectivity and persistence of certain pesticides; and (10) the role esterases, mixed-function oxidases, glutathione S-transferases and other enzymes play in the metabolism of a variety of insecticide chemicals and certain other pesticide chemicals. These studies are of importance in understanding the fundamental and practical aspects of pesticides biochemistry and pharmacology.

Chlorinated hydrocarbons. Workers at Vanderbilt University have studied the distribution of dieldrin after a single oral dose in rats and compared the results with a mathematical model set up by others. In the model, dieldrin levels in the brain are reduced promptly by redistribution to muscle and only after some time to fat. In experiments with rats, Hayes et al. found that dieldrin administered by stomach tube reached the highest brain levels in four hours and decreased gradually thereafter. The concentration in muscle remained essentially steady during 4 to 48 hours and there was no peak for muscle concentration that could be interpreted as replacing the peak for brain. On the basis of concentration or total amount in each organ, no reason was found to assign any special importance to muscle as a sink. The results indicate the danger of applying a mathematical model for one compound to another or from one organism to another.

DDT. Dr. Richard O'Brien (Cornell University) has been exploring the new technique of fluorescence correlation spectroscopy, which measures the fluidity of membrane interiors by following the fluctuation in intensity of fluorescent probes introduced into them. With lecithin membranes, he has been

able to show that DDT does not modify the fluidity, but does appear to expel the probe, suggesting an interesting model of how DDT might be involved in real membranes. Meanwhile, efforts have been made to improve the technique and extend its application to in vivo studies of cell processes. A modified fluorescence microscope set up has been constructed, which should permit the evaluation of fluidity studies in vesicles and allow use of briefer experiments (the earlier studies took seven hours for each run) and more diverse preparations.

DDT and dieldrin. Using rodents, Dr. Robert Jay (University of California, Davis) has attempted to determine the CNS basis for convulsions caused by DDT and dieldrin. He finds that changes induced by the compounds are indistinguishable from those caused by Metrazol. Through an analysis of evoked responses he has established that a major effect of dieldrin is to selectively increase cortical responses to sensory, transcallosal and direct cortical stimuli but not to affect responses to VL stimulation. Analysis of pyramidal tract activity confirmed that massive cortical cell discharge accompanies the enhancement of evoked responses. A most interesting finding is that sensorimotor cortex is made hyperexcitable to stimuli from all tested afferent pathways except the afferent pathway from the cerebellum that is relayed through the thalamic nucleus.

He has also established that recovery cycles of cortical cells are altered by chlorinated hydrocarbons. Recovery cycles of sensory cortices are shifted by dieldrin in a manner similar to that which occurs when the intensity of stimulation is increased 2 to 4-fold. The sensory activation pattern of motor cortex is completely changed, with a tremendous increase in responsivity developing at stimulus intervals of 20-40 msec. This is correlated with a similar excitability change in the pyramidal tract.

Dr. David Kupfer (Worcester Foundation) has continued studies on the estrogenic activity of chlorinated hydrocarbons. Since he has shown previously that ornithine decarboxylase (OD) is induced by estrogenic compounds, he has investigated the effect of chlorinated hydrocarbon insecticides on the OD activity in uteri of immature rats. Pretreatment with various doses of o,p'-DDT or E₂ (estradiol) caused a marked increase in the specific activity of OD in the 20,000g supernatant fraction of uterine homogenates but not in liver homogenates. Doses as low as 1 mg of o,p'-DDT or 0.25 ug of E₂ stimulated OD activity. The peaks of activity after o,p'-DDT and E₂ injections occurred at 6 and 4 hours, respectively. Treatment with inhibitors of protein synthesis effectively blocked the increase in OD activity caused by o,p'-DDT, indicating that the elevation in OD was due to enzyme induction. The comparative examination of various chlorinated hydrocarbon insecticides demonstrated that o,p'-DDT was the most potent inducer of OD activity in the uterus. The order of decreasing potency was o,p'-DDT, o,p'-DDD, p,p'-DDT, p,p'-DDD.

In somewhat related studies, Dr. William Kinter (Mount Desert Island Biological Laboratory) has continued investigations on DDT inhibition of avian shell and salt gland enzymes. He finds the domestic white Pekin duck to be an excellent model for investigation of the thin eggshell phenomenon, with about 20 percent thinning obtained when laying ducks are fed an

environmentally significant dose of DDE. Currently, pertinent shell gland enzymes, e.g., carbonic anhydrase, Ca^{++} binding protein, and a Ca^{++} activated ATPase, are being characterized in freeze-dried mucosal homogenates preparatory to DDE-inhibition studies in vivo and in vitro. DDE-feeding experiments with young ducks, both domestic white Pekins and wild type mallards, during adaptation to 60-100 percent sea water suggest that DDT-like pollutants might fatally disrupt salt and water balance in young sea birds.

The effects of selected organochlorine pesticides on linoleic acid peroxidation at 37° C was studied in order to determine if these pollutants could accelerate hydroperoxide formation in vitro. DDT, DDD, endrin, aldrin, dieldrin and chlordane at concentrations of 1.0 - 3.0 mg/ml caused 50-130 percent increases in the peroxide formation rate after two to four hours. The magnitude of this stimulatory effect was concentration dependent for all substances tested with the possible exception of aldrin. Clearly, organochlorine pesticides can function as free radical peroxidants in vitro, and since these pollutants are known to accumulate in the lipid rich regions of biological tissues in vivo, they are presumably present at the sites of lipid peroxidation. Hence, Dr. Kinter proposes a hitherto unsuspected mechanism of organochlorine toxicity, i.e., enhanced lipid peroxidation damage to subcellular elements such as membranes which is similar to the damage observed in oxygen toxicity and radiation injury.

Carbamates. Dr. Thomas Miller (University of California) has developed a highly sensitive heartbeat transducer for measuring the effects of bioactive amines and pesticides on the heartbeat of the cockroach. With only two heart chambers used for assay, the lower level of sensitivity for 5-hydroxytryptamine (5-HT) is $5 \times 10^{-8}\text{M}$ in a drop of saline. With this technique, it was found that 5-hydroxytryptamine is active directly on the myocardium whereas ACh is active exclusively on the cardiac neurons.

During Lindane poisoning, cardio-regulatory activity ceased at an early stage leaving an extremely regular heartbeat and indicated a lethal condition. Carbamate poisoning was accompanied by ordinary variations which gave no indication of lethal state since there was no difference between houseflies treated with lethal or sublethal doses.

Work in this laboratory has continued using also the flight motor activity of the housefly as an index of poisoning and kinetics of toxicity of carbamates and chlorinated hydrocarbons.

The flight motor potentials during carbamate poisoning reflect the lethal condition. In houseflies which recovered from threshold lethal doses, coordination between flight muscle units returned in a little over 40 minutes following topical abdominal N-thiomethylcarbofuran treatment. Those that did not recover never showed a return to coordination of the flight motor units for as long as potentials were recorded. Thus, it is evident that sublethal doses of N-thiomethylcarbofuran could be determined within the first hour of poisoning even though complete recovery of all overt behavioral activities might take several hours.

Penetration of ^{14}C -labelled carbofuran, leptophos and Monitor^R into the thoracic ganglion of the female housefly was measured while recording the muscle potentials from the top-most pair of dorso-longitudinal flight muscles. An accumulation of 25 pg in the thoracic ganglion was associated with carbofuran neurophysiological uncoupling, and convulsions developed within 10 minutes from topical treatment on the abdomen. By contrast, Monitor or leptophos did not cause uncoupling until 1,000 pg had accumulated at the central nervous system and the onset of convulsions took 30 to 40 minutes following treatment. Thus, the key finding was that phosphate insecticides are far less effective in disrupting the nervous system over a short time period than carbamates.

Organophosphates. Dr. Ullman Brady (University of Georgia) has developed a GLC method for analysis of acetylcholine involving demethylation. Results with the method are in agreement with published data using bioassay and has been used to determine the ACh in housefly heads at knock down with TEPP and dicotophos. He found that the ACh drops rapidly after treatment and is reduced by about 30 percent at knock down and by about 60 percent after 30 minutes. Thus the rapidity of disappearance of ACh appears to be related to rapidity of action of these, and perhaps other, pesticides.

Dr. Dorothy Woolley has continued comparisons of the time course of effects of parathion on behavior, brain electrical activity and tissue cholinesterase activity in mice, rats and monkeys. The interaction between parathion and atropine, methylatropine, scopolamine and methylscopolamine was investigated also in rats using the duration of phases of the maximal electroshock seizure, duration of postseizure depression, and grossly observable symptoms as end-points for indicating the antidotal effectiveness of the anticholinergic drugs.

These studies have shown that the rate of recovery of blood AChE activity is more rapid in mice than in rats and more rapid in rats than in monkeys after a single dose of parathion. Recovery of blood AChE is more rapid than that of brain. Recovery of brain electrical activity is also fairly rapid.

Comparisons of the anticholinergic drugs atropine and scopolamine, which act both centrally and peripherally, with methyl derivatives of atropine and scopolamine, which act primarily peripherally rather than centrally, show that scopolamine is highly effective in reversing parathion effects whereas methylscopolamine is ineffective. Results with atropine and its methyl derivative are not yet conclusive.

Resistance of houseflies to organophosphates has been known for about 20 years. Dr. Richard O'Brien (Cornell University) has found evidence that such resistance is attributable to a difference in the nature of acetylcholinesterase in resistant and susceptible houseflies. He has found a strain of highly resistant houseflies (more than 1,500 times as resistant to the organophosphate Rabon) and has demonstrated that its acetylcholinesterase is 206 times less sensitive to Rabon in vitro than the non-resistant strain. Furthermore, this ratio of 206 is compounded if a small increased sensitivity to phosphorylation (2.7-fold) is coupled with a 573-fold reduction in affinity of the enzyme for the agent. Cross-resistance to other

organophosphates is quite substantial and there is a rough correlation between the sensitivity of the enzyme and the degree of resistance.

Dr. O'Brien has also developed the theoretical kinetics and now completed the experimental procedures for the measurement of the two separate steps involved when an organophosphate reacts with acetylcholinesterase, i.e., a binding step followed by a phosphorylation step. The stopped-flow technique is now added to earlier and simpler spectrophotometric techniques and permits the measurement of these two steps in reactions that last only a few milliseconds. The procedure can now be applied even to the most highly reactive organophosphates.

Pesticide Metabolism and Interactions

Phosphate Insecticides. Dr. Robert Menzer (University of Maryland) has conducted a comparative study of the metabolism of phosphamidon, carbaryl and chlorotoluron by various tissue preparations of the rat and Japanese quail in order to determine the relative importance of various tissues in the metabolism of foreign compounds. In addition to liver, he has examined metabolism in lung, kidney and testis.

In the rat, carbaryl metabolism was greater in the liver microsomal fractions than in any other preparation. However, 1-naphthol was the major metabolite in all tissue fractions from the rat. In the quail, carbaryl metabolism was greater in the postmitochondrial fraction of liver in the overall quantity of 1-naphthyl-N-hydroxymethylcarbamate and 1-naphthol, which were the major metabolites in this tissue fraction. The major carbaryl metabolite in all other quail tissue fractions was 1-naphthol.

Although very little phosphamidon metabolism occurred in either animal, the metabolism in rat liver postmitochondrial supernatant was slightly higher with respect to the production of an unknown metabolite than that of the supernatant and microsomes combined. Phosphamidon metabolism in postmitochondrial preparations of quail liver was also higher with respect to the production of an unidentified metabolite than in liver microsomal and supernatant fractions. However, the production of the unidentified metabolite in postmitochondrial preparations of quail liver was not higher than that of microsomes and supernatant combined.

Chlorotoluron metabolism occurred only in the postmitochondrial fractions of quail liver. The major metabolites were N-(3-chloro-4-methylphenyl)-N'-methylurea and N-(3-chloro-4-hydroxymethylphenyl)-N'-methylurea. One compound was found which did not co-chromatograph with any of the available standards.

In addition to continued studies on organophosphate metabolism, Dr. Menzer has begun studies on animal metabolism of plant conjugates of pesticides. In the past year, efforts have been concentrated on developing procedures for radiosynthesis of 1-naphthyl glucoside and dimethoate-N-hydroxymethyl glucoside, so that the metabolism of these materials could be studied in animals. The synthesis of 1-naphthyl glucoside has been accomplished and the compound has been studied in some animal systems. The synthesis of the dimethoate derivative has not proceeded so smoothly. It is anticipated that

the compound will be made successfully within the next three months. Initial experience with these materials will determine the direction of this research over the next two years with these compounds and others.

Previous studies by Dr. Menzer showed that carbaryl is metabolized by human embryonic lung cells (HEL) in culture. The work has been extended to several more compounds of different chemical types. Results have been particularly useful for phenylurea herbicides since the mechanism of oxidative N-dealkylation was clarified in this system which was not possible in vivo because of the rate of metabolism and the production of only minute quantities of unstable intermediate metabolites.

Chlordimeform was quite susceptible to degradation by the HEL cell cultures. Over 97 percent of the applied chlordimeform was changed by the cells after 72 hours of incubation, with the major metabolites being N-formyl-4-chloro-o-toluidine (83 percent) and 4-chloro-o-toluidine (2.3 percent). Other minor metabolites included N-(4-chloro-o-tolyl)-N-methylformamidine and two unidentified compounds. When ¹⁴C-chlordimeform was incubated in medium without cells for 72 hours, the compound decomposed to N-formyl-4-chloro-o-toluidine (10 percent) with the remainder being unchanged. The formation of water-soluble metabolites was insignificant.

Metabolism of three ¹⁴C-labelled phenylurea herbicides, chlorotoluron, fluormeturon and metobromuron in HEL cells in culture was investigated. The metabolism of these three compounds occurred most rapidly within 12 to 24 hours of incubation. The oxidative metabolites which were formed during the first 24 hours were determined to be as follows: (1) for chlorotoluron: N-(3-chloro-4-methylphenyl)-N'-methylurea (1 percent), N-(3-chloro-4-methylphenyl)-N'-formylurea (0.7 percent), N-(3-chloro-4-methylphenyl)-N'-methylurea (1 percent), N'-(3-chloro-4-methylphenyl)urea (0.5 percent), and two unidentified metabolites (0.5 percent and 0.8 percent, respectively). Attempts to synthesize N-hydroxymethyl derivatives of the compound have not been successful.

Protein assays indicated normal growth of the HEL cells under the applied doses of the three compounds. Metabolites after incubations with HEL cell sonicates were found to be essentially the same as those from cells in culture.

Metabolism of chlorotoluron, fluormeturon, and metobromuron by liver microsomes from rats which were pretreated with three daily doses of sodium phenobarbital was investigated also. Results showed that the three compounds were quite susceptible to metabolism by the liver microsomes in contrast to the results of studies with HEL cells. After incubation with the microsomes and NADPH at 37° C for three hours, slightly over 60 percent of the applied chlorotoluron and fluormeturon formed oxidative metabolites, with 5 percent becoming water-soluble materials. Metobromuron was less susceptible to metabolism, approximately 85 percent of the applied dose remaining intact, with 12 percent being oxidative and 3 percent water-soluble metabolites. The oxidative metabolites were identified as follows: (1) chlorotoluron: N-(3-chloro-4-methylphenyl)-N'-methylurea (30.3 percent), N-(3-chloro-4-methylphenyl)urea (13.3 percent), and two unknowns (14 percent

and 2 percent, respectively); (2) fluormeturon: N-(3-trifluoromethylphenyl)-N'-methylurea (39.4 percent), N-(3-trifluoromethylphenyl)urea (8.2 percent), N-(3-trifluoromethylphenyl)-N'-hydroxymethylurea (8.1 percent), 3-trifluoromethylaniline (2.4 percent), unknown-1 (2.9 percent), unknown-2 (3 percent), and unknown-3 (2.3 percent). Work is continuing on the identification of the unknowns.

Parathion and methylparathion. In further studies on these compounds, Dr. Sheldon Murphy (Harvard University) has evidence that differences in the toxicity of the two compounds to fish may be explained on the basis of more rapid hydrolysis of methylparaaxon by fish liver homogenates. Hydrolysis was apparently glutathione dependent. A marked reduction in liver glutathione was observed following methylparathion, but not parathion, treatment. It was postulated that the detoxication of methylparathion is due to demethylation by the action of glutathione alkyltransferase. The role, if any, of the enzyme in the toxicity of the relatively new compound azinphosmethyl has not yet been determined.

Phosalone. Dr. Murphy and his colleagues began studies this year on phosalone, an organophosphorus insecticide with typical action as a cholinesterase inhibitor. Interest in the compound was stimulated by indications that phosalone is synergistic with several other organophosphates in acute toxicity tests. The potency of single doses as a carboxylesterase and cholinesterase inhibitor in mice has been determined. The dose for 50 percent inhibition of liver carboxylesterases, is approximately 3 mg/kg compared to a dose of 70 mg/kg that was required for 50 percent inhibition of brain cholinesterase. However, liver pseudocholinesterase activity was quite sensitive (5-10 mg/kg for 50 percent inhibition). Preliminary studies of oral administration in rats indicate a similar relatively high sensitivity of liver carboxylesterase and cholinesterase activity compared to brain cholinesterase. It is anticipated that further studies on phosalone in relation to its capacity to compete with sites of binding and/or enzymatic detoxification of other OP insecticides will help to further clarify the conditions under which synergism among OP insecticides may be anticipated.

Propanil. Dr. Murphy has extended his studies on the effects of organophosphates on hydrolysis of the herbicide propanil (3,4-dichloropropionanilide) to propionic acid. Investigations on the defoliant S,S,S-tributyl phosphotriothioate and the insecticides Abate and methyl parathion on liver propanil amidase, liver carboxylesterase and cholinesterase on propanil-induced methemoglobinemia indicate a dose-dependent inhibition of the enzymes and an induction of methemoglobinemia by DEF and Abate but little effect of methyl parathion.

Dr. Murphy and his colleagues previously provided indirect evidence that the methemoglobin producing action of the herbicide propanil was dependent upon its hydrolysis to form 3,4-dichloroaniline (DCA) which is further metabolized to an active methemoglobin producer by an SKF-525A-sensitive oxidative enzyme.

During the past year an *in vitro* system which allows more direct studies of the metabolic formation of an active methemoglobin producer from propanil or DCA was developed. Incubation with propanil or DCA in the presence of

hemoglobin as the target reactant allows quantitative measurements of both the hydrolytic (amidase) and the oxidative steps in propanil metabolism. This system has been applied to species comparisons of the metabolism of propanil and DCA in vitro as this relates to methemoglobin production by these compounds in vivo.

Intraperitoneal injections of 3,4-DCA produced about 70, 55 and 30 percent methemoglobin in either male and female rats, mice and guinea pigs, respectively, within 60 minutes. Production of methemoglobin with propanil was delayed in onset, presumably because it must first be hydrolyzed to 3,4-DCA. Measurements of hydrolysis of propanil by liver and kidney amidase revealed that female mice had significantly lower amidase activity in both tissues. This seems to explain why propanil produced less methemoglobin in female than male mice while no sex difference was observed for methemoglobin production by 3,4-DCA, which does not require hydrolysis.

Comparative toxicity of parathion and methyl parathion. Studies by Benke et al. at Harvard University demonstrated that sunfish are markedly more resistant to methyl parathion than to parathion while mice are about equally susceptible to both compounds. The reason for the differences in toxicity of the two compounds in fish is that methyl paraoxon is more rapidly hydrolyzed than paraoxon. However, the species differences in susceptibility was found to be due to differences in sensitivity of target cholinesterases, with the mouse enzyme being about 10-fold more sensitive than the fish enzyme.

The influence of age on toxicity and metabolism of the two compounds in rats was also investigated. It was found that there was a gradual decrease in susceptibility of animals up to the 63 days of the experiments. Changes in LD₅₀ values with age correlated with changes in rates of reactions leading to detoxication of both pesticides (metabolism of the oxygen analogs), which in turn correlated with binding of the oxygen analogs.

Potentiation of the toxicity of organophosphates. Further studies were carried out by Murphy et al. on the potentiation of toxicity by the carboxylesterase and pseudocholinesterase inhibitors S,S,S-tributyl phosphorotriothionate (DEF), the sodium salt of bis-dinitrophenylphosphate (BNPP) and triorthotolyl phosphate (TOTP). All of the compounds potentiated the toxicity of malathion, Guthion and Gutoxon to mice, though not to the same degree. Since Guthion and Gutoxon are not strong carboxylesterase inhibitors in contrast to other organophosphates reported to be potentiated by TOTP, quantitative predictions of potentiation based on relative carboxylesterase or cholinesterase sensitivity are of doubtful validity.

Cyclodienes. Dr. Frank Greene (Hershey Medical Center, University of Pennsylvania) has studied the metabolism and toxicity of cyclodienes, with special attention to species differences. Studies have been extended from the rat and dog to include the mouse and African Green monkey. As in the rat and dog, both aldrin and dieldrin were found to bind to cytochrome P-454 from mouse and monkey microsomes, producing Type I spectral changes. In contrast to results obtained in the rat, NADPH cytochrome c reductase was not inhibited by either aldrin or dieldrin in the male mouse. The enzyme from

female mouse liver was inhibited by aldrin but not by dieldrin. Results with microsomes from the African Green monkey were similar to those from female mouse liver.

Interesting results were obtained also in perinatal studies of rats fed dieldrin during the third week of pregnancy. Such treatment apparently resulted in permanent impairment of the competence of the offspring to metabolize dieldrin and the effect is passed on only through the exposed female to the next generation. The effect appears to be related to a defect in the terminal oxidase of the cytochrome P-450 system.

Another significant finding was that phenobarbital, 3-methylcholanthrene, or chronic administration of dieldrin did not induce higher metabolism of dieldrin, though the metabolism of typical substrates such as aniline, ethylmorphine and aldrin was stimulated. Moreover, dieldrin metabolism was not inhibited by carbon monoxide, suggesting that cytochrome P-450 is not involved in the formation of polar metabolites of dieldrin.

Mechanisms of insecticide metabolism. Dr. Robert Neal (Vanderbilt University) has continued studies on the molecular details of pesticide metabolism by microsomes. Using ^{35}S -labelled parathion he has been able to demonstrate that sulfur is bound to macromolecules of the microsomal membrane. Binding is increased by pretreatment of animals with phenobarbital or 3-methylcholanthrene. Most of the bound sulfur is detached from the remainder of the parathion molecule, apparently by cleavage by the mixed function oxidase system. The bound sulfur decreases the effective concentration of cytochrome P-450 in the microsomes and inhibits the subsequent metabolism of benzphetamine to some extent. Further studies on the effects of sulfur binding in vivo are currently under way.

Other studies indicate that the effects of inducers on the MFO system are confined only to increasing the amount, not the efficiency of cytochrome P-450 in microsomes. When the rate of metabolism of parathion was calculated on the basis of the concentration of cytochrome P-450 in rough-surfaced, smooth and whole microsomes there was no difference in the rates of metabolism by smooth and rough particles, but there was a decrease in whole microsomes from animals pretreated with inducers.

Dr. Raymond Harbison (Vanderbilt University) has continued studies on the prenatal effects of parathion as modified by inducers of hepatic microsomal enzyme activity. It was found that phenobarbital treatment of pregnant mice protected the conceptus against parathion. The treatment enhanced disappearance of parathion from plasma and reduced distribution to the placenta and other tissues. The observed phenomena have been related to phenobarbital acceleration of parathion oxidation and enhancement of paraoxonase activity.

By contrast, phenobarbital pretreatment potentiates the toxicity of chlorinated pesticides to the developing fetus. This is apparently due to the fact that metabolites of these compounds remain toxic and can traverse the placental barrier.

Dr. Christopher Wilkinson (Cornell University) has continued studies on the properties of microsomal enzymes from insects with particular reference to degradation of insect juvenile hormone (JH). In view of the importance of JH in determining insect development, a great deal of interest has recently been focused on a number of synthetic compounds which mimic the action of this hormone and are therefore of considerable potential in insect control. Some synthetic JH mimics are effective inhibitors of epoxide hydrase activity. Thus their mode of action might be due in part to their ability to stabilize endogenous JH and to consequently disrupt development. Studies were initiated to characterize epoxide hydrases in insects and to evaluate a variety of inhibitors. It now seems clear that there are probably several different epoxide hydrases with different substrate specificities and varying degrees of susceptibility to inhibitors. Of particular interest is the finding that many of the inhibitors of the oxidative P-450 mediated microsomal reactions (1,3-benzodioxoles, 1,2,3-benzothiadiazoles, substituted imidazoles, etc.) are also inhibitors of the epoxide hydrase system. The latter is obviously closely related to P-450 but is not inhibited by CO and will proceed under anaerobic conditions. The relationship between these two systems is also very interesting in view of the importance of each in the activation and degradation of carcinogenic compounds in mammals. A comprehensive study of epoxide hydrases in insects will be an important component of future studies.

Dr. George Webb (University of Vermont) has studied the effect of several chlorinated hydrocarbons on cation transport and ATPase. In general, the insecticides tested had only slight effects on the rate of active transport of sodium (the short-circuit current) or the passive ion permeability of isolated frog skin. DDT appeared to stimulate active transport to some degree, heptachlor increased passive ion permeability while both alpha and gamma chlordane greatly decreased passive ion permeability. Methoxychlor had very little effect on either short circuit current or skin resistance whereas lindane produced a reversible decrease in the short circuit current.

EPN. Dr. John Hutchens (University of Chicago) has studied the effects of enzyme inducers on the toxicity of EPN and of EPN on inducible systems. He finds that both DDT and phenobarbital strongly induce arylamidase, N-demethylase and detoxification of EPN. Hepatic and serum diethylsuccinase and tributyrinase are induced to a lesser extent. Brain cholinesterase is not induced at the doses tested.

EPN was found to strongly inhibit the serum aliesterases and N-demethylase but had little effect on the EPN detoxication system, brain or red blood cell cholinesterase or the hepatic esterases. A potentially important finding was that serum tributyrinase is among the most sensitive enzymes to EPN. It was inhibited almost totally at EPN levels that do not affect brain or red blood cell cholinesterase. Since man has considerable serum tributyrinase, it might be feasible to use this enzyme as a more sensitive assay than the conventional red blood cell cholinesterase assay for detection of exposure to phosphorothioate insecticides.

Because of the opposite action of EPN and inducing compounds, combinations were tested. At the same levels of induction, DDT and phenobarbital-induced enzymes behave differently. EPN suppresses induction by phenobarbital but not that by DDT.

Pesticides Resistance and Tolerance

Endrin. A comparative study of *in vivo* endrin metabolism with endrin susceptible (S) and endrin resistant (R) pine mice has been made by Dr. Ryland Webb (Virginia Polytechnic Institute). It appears that S mice excrete a greater amount of the radioactivity from ^{14}C -labelled endrin than R mice in the form of a water soluble metabolite, though total urinary excretion is approximately equal between the two strains. On the other hand, results to date indicate that a greater percentage of the label is retained in the brain and adrenal tissues of S mice and that there is an approximate two-fold greater demethylase activity in the R strain. The major endrin metabolite excreted has been isolated and tentatively identified as the 9-OH derivative.

DDT. Dr. Leon C. Terriere (Oregon State University) has continued studies begun two years ago on the biochemical-genetic basis of resistance of houseflies to DDT. He has obtained substrains from a cross between his Fc strain, highly resistant to DDT and other insecticides through oxidative mechanisms, and a susceptible strain carrying morphological markers for each of the five autosomes of the housefly. Biochemical studies on the substrains show that the oxidase of chromosome II, which is responsible for the metabolism of compounds such as baygon and aldrin, has no genetic relationship with the oxidase of chromosome V which attacks DDT. It appears also that more than two chromosomes are responsible for full expression of the mixed function oxidase activity of this strain.

Dr. James Yarbrough (Mississippi State University) is investigating the mechanisms of resistance of mosquitofish to DDT, endrin and other chlorinated hydrocarbons. The amount of DDT converted to DDD in resistant fish is significantly greater than in susceptible fish livers and there is significantly more DDE in susceptible than in resistant fish. No data are available on the relative toxicity levels of either DDD or DDE on susceptible and resistant mosquitofish, so that the differences seen in conversion of DDT to either DDD or DDE can not be related directly to toxicity at present.

In work with endrin, comparisons were made between fish exhibiting symptoms of poisoning and those not exhibiting symptoms at the time of sacrifice. Symptoms of poisoning correlated within a population with higher tissue endrin concentrations. In general, there were lower endrin concentrations in tissues of symptomatic S fish than in tissues of asymptomatic S fish. This was the reverse of what was seen in R fish.

The higher endrin concentrations in the S as compared to the R fish suggests that there is an effective barrier in the R population. However, there does not appear to be a functional barrier in the more tolerant S fish as compared to the less tolerant S fish. Apparently the S fish tolerance is related to factors other than a membrane barrier.

Mitochondria were prepared from resistant and susceptible fish livers and incubated in an isotonic medium (0.32 sucrose) with Ca-45 in a series of varying p,p'-DDT concentrations. A definite difference was found in the uptake of calcium between control mitochondrial preparations from susceptible and resistant mosquitofish. DDT exposure at all levels had little if any effect on the calcium uptake in resistant preparations. However, it greatly effected calcium uptake in susceptible preparations.

Comparison of susceptible (S) and resistant (R) fish, using liver microsomes, revealed that neither the O-demethylation of nitroaniline nor the N-demethylation of nitrophenyl dimethylcarbamate was significantly different between the two strains. The epoxidation of aldrin to dieldrin, however, was about 50 percent faster in the R strain.

Unexpectedly, microsomes from R livers contained only about half as much cytochrome P-450, the terminal oxidase in the microsomal electron transport chain, as did those from S livers.

Metals

Metallothionein. Dr. David Natusch (University of Illinois) has continued investigations to gain a detailed understanding of the structure of the cadmium binding protein, metallothionein. In particular, he has examined the relationship of structure of the protein to the presence of Cd^{2+} and Zn^{2+} (the main metal components found in vivo) and other metal ions, e.g., Cu^{2+} .

Research goals for this year included the development of a large scale isolation and purification procedure for horse kidney metallothionein; the establishment of a routine analytical protocol for determining metal content, total protein and protein purity and for reversible extraction of nascent metal ions; and the initiation of extensive nmr studies of pure metallothionein. Isolation and purification procedures based on sephadex chromatography and ion exchange have been developed and have made possible isolation of metallothionein at the rate of about 25 mg/week. Atomic absorption spectroscopy is used routinely for precise Cd and Zn determination and initial investigations of energy dispersive X-ray fluorescence spectroscopy for multielemental analysis have been conducted. Gel electrophoresis gives one major band for metallothionein with a very minor impurity, which Dr. Natusch is attempting to eliminate.

Lead. Dr. Raymond Clasen (Rush Presbyterian St. Luke's Medical Center) has attempted to measure the effects of drugs on the edema of lead encephalopathy in the rat and to develop the experimental model of lead encephalopathy in the monkey for use in psychological testing.

Experiments with intraperitoneal hypertonic urea were performed on three different groups of rats for a total of 10 lead-poisoned animals, six lead-poisoned animals treated with urea, 10 control animals and 13 control animals treated with urea. The mean water content of the control was 78.8 percent. Controls given urea had a mean value of 77.2 percent. Lead-poisoned animals given the urea had a mean value of 76.4 percent while in the untreated

lead-poisoned animals it was 79.3 percent. From this it is concluded that intraperitoneal urea dehydrates the central nervous system in both normal and lead-poisoned rats.

The results with steroids are less clear. In one completed experiment involving 16 lead-poisoned animals, one-half of which received decadron, there would appear to be a suppression of edema by the steroid. However, subsequent experiments involving 24 lead-poisoned animals have failed to show any difference between the groups. Thus at the present, a definite statement on the effects of steroid treatment on the edema of lead encephalopathy is premature. The probabilities, however, seem to be that it is not effective.

Experiments were also conducted with the chelating agents, BAL and EDTA, given in combination. These experiments involved four lead-poisoned animals and four controls. Results on water uptake indicate no effect on the poisoned animals or the controls.

Dr. Walter Farkas (University of Tennessee Memorial Research Center) has discovered that lead is a potent catalyst for the depolymerization of RNA. Thus a major objective now is to learn if Pb^{2+} -catalyzed depolymerization of RNA could account for the extreme toxicity of Pb^{2+} towards the biosynthesis of hemoglobin. A study of the effects of Pb^{2+} on transfer RNA has shown that whereas both the amino acid acceptor function and the capacity of aminoacyl tRNA to bind to ribosomes in response to specific codons is impaired, the Pb^{2+} concentrations at which these effects are observed is too high to account for the sensitivity of the hemoglobin synthesizing apparatus towards Pb^{2+} . Transfer RNA is largely double stranded and it has been shown that double stranded RNA is much more refractory to the depolymerizing action of Pb^{2+} than single stranded RNA. Messenger RNA, although it does have more secondary structure than was originally predicted for it, is much more single stranded than tRNA. Therefore, Dr. Farkas is now examining the effects of Pb^{2+} on synthetic and natural mRNA's. The experiments with synthetic messengers, such as polyuridylic acid and polyadenylic acid, have revealed similarities to the work with tRNA; namely, that there is a threshold concentration of Pb^{2+} below which Pb^{2+} will not impair biochemical function of RNA and that the rate of loss of messenger function is initially rapid, then slows down and, finally, there is a small fraction of RNA that is refractory to the action of Pb^{2+} .

The studies with natural messengers show that they are considerably more sensitive to Pb^{2+} . This is not unexpected since a single break in any of the phosphodiester bonds of a natural mRNA would render it inactive whereas a break in poly U or poly A would merely result in the production of smaller oligopeptides of phenylalanine and lysine respectively.

Recently, it was shown by others that in patients suffering from plumbism the biosynthesis of alpha globin chains is depressed to a greater extent than is beta chain production. Dr. Farkas has been able to confirm this observation using rabbit reticulocytes, but the degree of inhibition of alpha chain synthesis is much less than in human red cells. This may be due to species differences or to the possibility that Pb^{2+} exerts its effect on hematopoiesis

at the level of erythrocyte precursor cells in the bone marrow but not in the circulating reticulocyte. It may in fact be that the synthesis of the mRNA for alpha chains is impaired to a greater extent than is the synthesis of the mRNA for beta chains.

Dr. Albert Krall (Medical University of South Carolina) has conducted studies to determine how lead inhibits the activity of kidney mitochondria and how Mg or other ions interact with and affects lead-induced inhibition. He found that rats fed a Mg-deficient diet gain little or no weight. Removal of 90 percent of the calcium from the diet allows them to gain weight at the same rate as the controls for 10 days, after which time they gain essentially no more weight. Addition of lead to the simple magnesium deficient diet stops any weight gain observed with that diet. Addition of lead to the low calcium, magnesium deficient diet completely stops weight gain by the second day on the diet. Rats on the magnesium deficient diet survive about 18 days; those on that diet to which lead is added survive about the same length of time. Rats on the low calcium, magnesium deficient diet survive 36 days and with addition of lead a few rats are alive at 46 days. Subjectively, those rats fed lead do not seem to exhibit as flagrant a reaction to magnesium deficiency in either group.

All the rats in the magnesium deficient, lead-fed group exhibited a wild running, tonic-clonic seizure pattern in response to changes in position at about twelve days after initiation of the diet. Most of the magnesium deficient animals also showed some seizure activity about two days later than the lead-fed animals, but the severity of the seizures was not as great nor did they occur in all animals.

Assays of kidney mitochondrial properties--respiratory control ratios and P/O ratios--showed that about the same degree of deterioration of mitochondrial integrity occurs after five days of lead feeding to the low calcium, magnesium deficient rats as occurs after six to twelve weeks of lead feeding to rats on a control diet.

The interrelationship between lead and calcium and magnesium metabolism being explored indicates that absence of the latter two ions from the diet should make lead ingestion much more toxic. In this connection, Dr. Krall thinks it may be significant that lead screening assays in the city of Charleston show that as high as 40 percent of children living on the Charleston water supply contains only 7.0 mg per liter calcium and 0.85 mg/liter magnesium, less than ten percent of that found in adjacent localities which draw water from deep wells. Thus it may be that mineral content of the diet may alleviate lead poisoning.

Dr. Raymond D. Harbison (Vanderbilt University) has continued studies of lead toxicity and distribution in newborn and adult rats. Groups of newborn and adult rats were injected intraperitoneally with Pb acetate. Female rats were found to be the most sensitive to lead toxicity. At a dosage of 300 mg/kg, a 30 percent incidence of death was noted. No deaths were recorded in adult male or newborn at this dosage. However, the newborn appeared more sensitive to lead toxicity when compared to the adult male.

Thus, significant lead toxicity differences are seen between age and sex groups. Lead distribution studies indicate lead concentrates differently in the newborn. Significantly greater concentrations are seen in newborn brain and lung tissue. This may account for the increased frequency of lead encephalopathy and the depression of the reticuloendothelial system in the newborn. The data suggest that lead toxicity standards cannot be generalized between sexes and age groups if the rat is indeed a representative model for the human.

Dr. William Buck (Iowa State University) has studied the effect of lead on the antibody response of pigs. He found that the antibody response of pigs to flagellar antigen of Salmonella choleraesuis was significantly greater in control animals as compared to those exposed to lead. There was no appreciable difference in response to somatic antigen. This differential nature of the responses to the two antigens may reflect the site of action of lead on the immunologic system.

Results using hamsters were similar to those in pigs. Although at least an LD₅₀ dose of S. typhimurium was given, only minimal illness and deaths were produced.

Mercury. Dr. Robert Bach (Wayne State University) has continued studies on the chemical behavior of organomercury compounds. He has used the Extended Huckel Molecular Orbital Theory to investigate the question of why dimethylmercury(I) disproportionates into dimethylmercury(II) and mercury metal. He finds that as the covalency of the mercury-ligand bond increases the energy of the Hg-Hg bonding molecular orbital is increased and thus is more susceptible to radical cleavage or attack by electrophiles. This is a result of the increasing p character in the Hg-Hg bond as the covalency of the mercury-ligand bond increases. Thus, the enzymatic methylation of the mercurous salt would afford dimethylmercury and elemental mercury.

Using methyl (methylthio) mercury ($\text{CH}_3\text{HgSCH}_3$) as a model compound, the exchange reaction of mercaptans with the methylmercury moiety has been studied. This compound has presumably been isolated from shellfish and its vibrational spectral properties have been studied. However, the chemical reactivity of this class of compounds has not been studied. The results obtained thus far provide dramatic evidence for rapid exchange of sulfhydryl groups bonded to methylmercury. The data provide a plausible mechanism for the facile migration of CH_3Hg in living systems. These studies are being extended to include other ligands of biological significance.

Cadmium. Dr. Tom Miya (Purdue Research Foundation) has studied the interactions and consequences of interactions between Cd and drugs. He has found that Cd potentiates hexobarbital sleep time. The minimal dose for effect is 2 mg/kg in male albino rats. In addition, it was shown that a single dose of Cd acetate continues to potentiate the hexobarbital effect for at least 10 days.

Cd was found also to potentiate the effects of tremorine in rats. Tremorine was chosen as a model compound because its pharmacological activity is

dependent not upon the parent compound, but upon its oxidative metabolite, oxotremorine. Cadmium treatment caused a two-fold increase in the onset time for tremors development.

To determine by what mechanism cadmium potentiates drug action, the influence of cadmium on the plasma decline of hexobarbital and zoxazolamine was studied. Though Cd treatment significantly prolonged the hexobarbital sleep time, it did not influence the wakening plasma levels of the drug. These data suggest that CNS sensitivity to hexobarbital was not altered. Plasma levels of hexobarbital were significantly increased in the cadmium-treated rats sacrificed at the mean duration of sleep time observed for the control rats. This suggests that Cd interferes with the plasma decline rate for hexobarbital and thus prolongs hexobarbital sleep time.

Cadmium also significantly prolongs zoxazolamine-induced paralysis time. Plasma levels of the drug determined upon recovery from paralysis were higher in control rats than in cadmium-treated animals. This difference in levels, although small in magnitude, indicates a possible change in the tissue sensitivity to zoxazolamine. In contrast, the zoxazolamine levels in the plasma of Cd-treated rats which were sacrificed at the mean paralysis time of the controls were significantly higher than in the corresponding control rats. To determine the mechanism by which Cd prolonged the plasma decline rates of hexobarbital and zoxazolamine, the influence of Cd on the in vitro metabolism of these two compounds was studied. Three day pretreatment of rats with Cd was found to significantly prolong the in vitro metabolism of hexobarbital in rat liver homogenates. Data for zoxazolamine metabolism is not yet available.

Metal binding. Dr. Sara Bryan (University of New Orleans) has continued her studies on induction of metallothionein by Cd and Hg, and binding of metals by nuclear constituents. She reports that nuclei isolated from mercury-challenged mice contained approximately 20 times as much mercury as those from controls. She has fractionated the nuclei into heterochromatin and euchromatin fractions. A subsequent analysis of the mercury content of each fraction revealed to localization of mercury in euchromatin (the "active" component) in controls with a three-fold enrichment in mercury-treated mice.

Other studies revealed a localization of copper in the heterochromatin fraction in challenged mice. There was a rather variable distribution of metal in controls, with euchromatin showing a level of approximately 0.3 ppm/mg DNA accumulation of copper in this (the euchromatin) fraction. The data support the hypothesis that heterochromatin may function in a protective capacity against copper toxicity in mice.

Soluble RNA polymerase is inhibited 50 to 60 percent in copper-treated rats while the pellet obtained following solubilization, which shows essentially no activity in the control, retained appreciable activity in the copper-treated material. This differential effect suggests that copper binds to the nuclei in a manner such that the enzyme remained "fixed" in the pellet and thereby prohibits normal solubilization. Furthermore, the portion that is solubilized is inactivated as evidenced by an elevation in protein and a decrease in specific activity.

Dr. Albey Reiner (University of Massachusetts) has studied the mechanism of resistance of Escherichia coli to heavy metals. Several hundred clinical isolates of E. coli were tested before and after their exposure to F-factors for their abilities to transfer to a standard E. coli strain plasmids which confer resistance to mercury, cadmium and silver cations, methyl mercury or phenyl mercuric acetate. Transferable resistance to all except silver was found, with many different resistance patterns (e.g., phenylmercuric acetate only, methyl mercury and mercury cations only, mercury cations and cadmium cations only, etc.). Resistance was often, but not always, accompanied by resistance to one or more antibiotics.

Attention was focused on the mechanism of resistance to mercury cations. In each instance tested, resistance was found to be accompanied by a net removal (volatilization) of mercury from the medium. Resistance and net loss of mercury were shown to be inducible. Work is continuing on the problems of resistance to cadmium cations and organic mercury compounds. In these instances the heavy metal is not volatilized.

Natural Products

Aflatoxins. Dr. Donald Lee (Oregon State University) has continued studies on the metabolism of aflatoxin B₁ in rainbow trout and on the relative carcinogenicity of aflatoxins B₁, M₁ and B_{2a}. Emphasis was given this year to characterization of metabolites of B₁ formed in vitro in liver.

A major metabolite formed from aflatoxin B₁ by trout liver was identified as aflatoxicol (aflatoxin Ro). This reduced form of the toxin was identified by IR, TLC chromatography, UV absorption and mass spectrometry. The organic synthesis of Ro was developed and the isomers (designated Ro Rear and Ro Front, according to their migration on a thin layer plate) were separated by column chromatography. RoF had R_f values identical with the naturally occurring compound. The toxicity of RoF to rainbow trout is much greater than that of RoR. Acute toxicity studies indicate its LD₅₀ is approximately 1.2 mg/kg body wt. of trout. No deaths occurred when RoR was given (IP) at 1.8 mg/kg. A feeding trial to determine the carcinogenicity of these compounds is underway.

The transformation of aflatoxin B₁ to a compound extremely toxic to Bacillus subtilis was shown to occur when the mycotoxin was incubated with isolated trout liver microsomes. The reaction required NADPH, oxygen and appeared to be associated with oxidative drug metabolism. A very polar fluorescent compound seen on TLC plates was associated with toxicity.

A feeding trial in which B_{2a} was fed to trout at 256, 512 and 1024 ppb for 12 months produced no tumors. Some fish were held for eight months after the toxic diets were terminated. Thus it appears that alteration of the difuran moiety of the molecule completely **destroys** the carcinogenic properties of aflatoxin B₁.

Other studies show that aflatoxin B₁ must be converted to a reactive compound before toxicity occurs. Because the rainbow trout is so sensitive to

aflatoxin, comparative studies with more resistant species, or strains, of trout should be helpful in understanding the mechanism of toxicity and carcinogenesis.

Dr. R. B. Alfin-Slater (University of California) has studied the modification of aflatoxin toxicity by dietary factors. Seven groups of rats were divided into corn oil-fed and fat-free groups and each of these subdivided into vitamin E-containing and E-free diets, each with or without aflatoxin at 1.7 ppm for three months. The animals were sacrificed after three months. It was found that aflatoxin ingestion depressed growth significantly in the fat-free and fat-fed groups. A slight decline in weight observed in the corn oil fed groups was not statistically significant. No grossly apparent tumors were observed in these animals and histological examination did not reveal any pathological changes. Liver weights paralleled increases in weight gain observed on high fat diets, but when expressed as percent of body weight, a statistically significant increase due to aflatoxin treatment was evident on all diets. Kidney weights generally decreased in aflatoxin-treated animals. No significant changes were observed in other tissues. Other than characteristic effects of fat free diet (increases in liver cholesterol and total lipid) no significant changes occurred in the liver as a result of aflatoxin ingestion. No significant changes occurred in the plasma of corn oil-fed rats; however, there was an increase in the plasma cholesterol levels of the fat-free group and a decrease in the high lard-fed group due to aflatoxin treatment. The vitamin E-free groups showed no tocopherol present in plasma. Groups supplemented with dietary tocopherol showed a progressive decrease as the fat content of the diet increased (particularly with corn oil) and, in addition, there was a decrease of plasma tocopherol in the aflatoxin-fed rats.

All livers were analyzed but very few significant changes in fatty acid distribution have been observed other than those relating to the diet (i.e., increases in monounsaturated and 20.3 fatty acids on a fat free diet.).

Rats for long-term studies were started on the experimental diets between August and October 1973. The fat-free group reached 50 percent mortality in May, and in June 1974 for the vitamin E-deficient and vitamin E-sufficient group, respectively. The 30 percent corn oil, vitamin E free group also reached 50 percent mortality approximately seven months following the conclusion of aflatoxin administration. These groups have been sacrificed and the rest of the animals are being sacrificed. Fatty acid and cholesterol biosynthesis studies in rats treated with aflatoxin have revealed increases in the manufacture of these lipids in liver.

Dr. Colin Campbell (Virginia Polytechnic Institute) has continued studies on the metabolism of aflatoxins in rats, monkeys and humans. AFP_1 , which is the O-demethylated derivative of AFB_1 , has been shown to be a major excretory product (20 percent) when rats or monkeys are injected with AFB_1 . Repeated attempts have been made to determine whether it also represents a major route of metabolism and excretion in the human. Although it may be produced in small quantities by human liver microsomes in vitro, it could not be found in the urine of human subjects known to have consumed levels of AFB_1 sufficient to produce AFM_1 . It was not present as the free phenol or as the sulfate or

glucuronide conjugate. Thus it does not appear that AFP₁ represents a major metabolite in the human for ingested AFB₁.

The continuation of earlier studies designed to determine whether AFB₁ affects the mitochondrial functions of electron transport and transcription have indicated that some type of metabolic activation seems to be required for maximal effect. For example, mitochondria isolated from rats injected i.p. with 5.3 mg/kg body weight four hours prior to sacrifice exhibited a 30 to 40 percent reduction in UTP incorporation compared to 11 percent maximal inhibition when AFB₁ is added in vitro. The preliminary studies showing AFB₁ inhibition of electron transport have been more clearly defined and show a specific site of inhibition located between cytochrome b and c(c₁). Moreover, the penetrability of AFB₁ to the inner mitochondrial membrane appears to be involved, since it is possible to achieve 63 percent inhibition with submitochondrial particles and only 45 percent inhibition with intact mitochondria.

Cnidarian toxins. Studies on the chemical nature and mode of action of Cnidarian toxins were continued by Dr. Richard Blanquet (Georgetown University). Methods for nematocyst isolation and purification of nematocyst toxins is essentially complete. These methods have proved successful on two other species of cnidarians, Cyanea capillata and Stomolophus meleagris. Isoelectric focusing techniques have allowed a more complete analysis of Chrysaora toxic. Several toxin proteins were revealed with isoelectric points at the following pH values: 9.7, 8.2 (minor component) 7.2 (minor component), 4.3, and 3.5. The latter two proteins are consistent with an earlier report of high concentrations of acidic amino acids in at least one toxic protein.

Initial efforts to determine the mode of action of the toxins involved the effect of Chrysaora whole toxin on mammalian heart function through the use of anesthetized rats. Toxin was administered via jugular cannulation either in small consecutive doses or in a single dose equal to the total amount of toxin administered as consecutive doses. Electrocardiograms of the hearts of injected rats demonstrated a distinct bradycardia, lowered blood pressure, prolonged P-R intervals, increased Q-R amplitude, T wave enlargement, and intermittent atrioventricular blockage.

The bradycardia is thought to be associated with a progressive decrease in the extent of ventricular relaxation with each successive cardiac cycle. The toxin may be a direct constrictor of vascular smooth muscle. Prolonged P-R intervals are indicative of first degree A-V block and denotes a disturbance of the A-V conduction pathway. Irregularities in the QRS complex indicates a block somewhere in the normal conduction pathway through the ventricles. Elevated T waves may evidence a prolonged depolarization of the ventricles. It is interesting to note that in those studies where pre-injection levels of heart rate and blood pressure were reinstated, the P-R interval remained prolonged and the T wave stayed enlarged. This may indicate some lasting damage to the cardiac conducting system.

During the course of experimentation on the neuromuscular effects of Chrysaora toxin, it was reported that frog gastrocnemius muscle exposed to Chrysaora

toxin and then repetitively stimulated, fatigued in a contracted state rather than the relaxed state demonstrated by controls. This led to investigation of the effects of cnidarian toxins on the activity of muscle ATPase activity. As Chrysaora was not available during the winter months, Stomolophus meleagris, a potent stinger was used in its place. Experiments were performed using myosin ATPase extracted from the lateral muscle of trout and the measurement of ATPase activity via the release of phosphate from ATPase as measured by a modification of the method of Fiske & Subbarrow.

Isolation of the nematocyst contents of the jellyfish Stomolophus revealed the presence of four protein fractions (I, II, III, IV). When each fraction was tested individually, only fractions II and IV possessed ATPase activity: Fraction II demonstrated considerable activity with an average value of 8.3 ug Pi/mg protein/min. Fraction IV was slightly higher at 10.7 ug Pi/mg protein/min. When these fractions were added to myosin ATPase extracts, the total activity was essentially additive, though in some cases a slight synergism was noted.

Fatigue in the contracted state may thus be due to a decrease in ATPase availability leading to the increase of rigor complexes in the muscle. Studies will be extended to include Chrysaora toxin and to determine kinetic parameters of Myosin ATPase in the presence and absence of nematocyst toxins. The effect of these toxins on Ca^{++} permeability is also planned.

Studies last year demonstrated that Chrysaora toxin was ineffective in releasing histamine from rat whole blood and leucocytes. Experiments using mast cells isolated from rat peritoneal fluid, however, showed that this toxin was consistently effective in releasing significant amounts of histamine when compared to various controls and compound 48/80, a potent histamine releaser. Histamine release by the toxin was unaffected by dialysis of whole toxin or freezing but was destroyed by boiling or by protein denaturants. Histamine releasing activity was also lost after separation of the protein components and was not regained on combining the fractions. The cause is not known but may involve modification of the active component during separation and purification.

Studies are underway on the toxin's ability to act as a kinin or to release kinin-like components from blood.

Similar studies on toxins from the jellyfish Stomolophus meleagris have been conducted by Dr. Paul Toom (University of Southern Mississippi) using both rabbits and rats as experimental animals. Electrocardiographic recordings of these animals exhibited numerous abnormalities including: inversion of the P and T waves, alterations of the QRS complex, bradycardia, and atrioventricular block. Plasma samples were characterized by hemolysis, hyponetemia and hyperkalemia. When other animals were injected with sublethal doses of the toxin, serum enzyme and isoenzyme analyses suggested that the toxin did not produce cardiac tissue damage. Thus, the main conclusion from this study was that the cardiac effects of the toxin are due to disruption of myocardial conduction rather than to direct cardiac tissue damage.

The effect of Stomolophus meleagris toxin on sodium transport across isolated frog skin was investigated. The toxin was found to initially stimulate sodium transport. Initial stimulation was then followed by an inhibitory action.

Subsequently, the toxin was fractionated into five fractions, two of which were found to increase active sodium transport. One fraction was found which inhibited active transport of sodium. Inhibitor studies with Ca^{++} , ouabain, etc. suggested that the activating process was probably due to a component of the venom which increased the permeability of the membrane to sodium, while the inhibiting action involved a component which acted as an inhibitor of $\text{Na}^{+} - \text{K}^{+}$ ATPase.

Since studies on intact frog skin suggested that the toxin has at least one component which interacted directly with $\text{Na}^{+} - \text{K}^{+}$ ATPase within the membrane, studies were initiated to study the effects of both whole and fractionated toxin on $\text{Na}^{+} - \text{K}^{+}$ ATPase in vitro. When ATPase was partially purified from rat brain, low concentrations of toxin were found to activate both $\text{Na}^{+} - \text{K}^{+}$ ATPase and mitochondrial ATPase about 100 percent, while higher toxin concentrations (>10 ug/ml) inhibited these two ATPases about 50 percent. On the other hand, oligomycin insensitive ATPase was inhibited at low toxin concentrations and activated at toxin concentrations above 8 ug/ml. While this study is still underway, it now appears that fractionation by membrane partition chromatography will give 4 distinct fractions which inhibit (ca 30 percent) Na-K-ATPase and one fraction which has no effect on this ATPase. Of these five fractions, three also inhibit mitochondrial ATPase (65 to 80 percent), one fraction activates (45 percent) and the fifth has no effect on this ATPase. When these fractions are tested on oligomycin insensitive ATPase, two have no effect, two show a slight activating effect (10 to 30 percent) while the fifth inhibits the enzyme about 65 percent.

Gaseous Pollutants

Ozone. Dr. Bernard Goldstein (New York University Medical Center) has continued to evaluate his previous observations that a primary mechanism of ozone toxicity is the breakdown of unsaturated fatty acids (UFA) in cellular membranes with the concomitant formation of free radicals, peroxides, and carbonyl compounds. Microchemical techniques have been employed for the determination of breakdown products so as to establish parameters indicating exposure to relatively low levels of ozone. The most promising technique evaluated in the past year has been the spectrofluorescent procedure of Tappel, in which the linkage to protein of carbonyl compounds is detected by characteristic fluorescence emission and excitation spectra. Evaluation of sera obtained from rats and rabbits exposed to 6-8 ppm ozone for 2-4 hours demonstrated fluorescence spectra similar to sera directly ozonized or to which extracts of ozonized UFA had been added. Further specificity has been achieved by evaluating the fluorescence characteristics of albumin extracted from serum. This procedure utilizes a specific protein and takes advantage of the fact that fatty acids tend to circulate in serum bound to albumin and are extracted with albumin using standard techniques. Of note is that there is also a readily detectable decrease in native protein fluorescence of albumin following ozone exposure which presumably represents an effect on

tryptophan molecules in the extract. The major technological problem remaining prior to assessment of this technique at relatively low ozone concentrations has been some difficulty in reproducibly extracting serum albumin without artifactual lipid peroxidation from control samples.

In the past year, it was demonstrated that inducing regimens of various compounds including para-aminobenzoic acid, chlorpromazine, and phenobarbital result in modification of ozone toxicity. Phenobarbital, an inducer of cytochrome P-450, potentiates ozone toxicity while allylisopropylacetamide (AIA), a related compound which reduces cytochrome P-450 levels, protects against the lethal effects of ozone. Of note is that AIA is believed to destroy cytochrome P-450 by way of an epoxide intermediate and that lipid peroxides also decrease cytochrome P-450. Furthermore, the heme-containing cytochromes potentiate lipid peroxidation. These studies have led to the hypotheses that cytochrome levels play a role in ozone toxicity and that the phenomenon of tolerance is due to an ozone-induced decrease in cytochrome levels produced by the initial sublethal ozone exposure.

An additional study revealed wide differences in the response of 22 inbred strains of mice to lethal levels of ozone. In at least one case, resistance to ozone is inherited as a dominant characteristic. The response to nitrogen dioxide appears to be similar to that of ozone.

SO₂. Dr. Yves Alarie (University of Pittsburgh) has continued efforts toward quantitative evaluation of sensory irritation by SO₂ (and other airborne chemicals) in industrial situations, and urban atmospheres and toward determination of the mechanism of desensitization with repeated exposures. Using mice exposed to SO₂ to evaluate sensory irritation of the upper respiratory tract, it was found that when abrupt changes in exposure concentrations were made (0 to 100 ppm in one minute) the reaction is much more pronounced than when slow changes are made (0 to 100 ppm in one hour). In the latter case a very small reaction is seen. This phenomenon has been observed in humans exposed to SO₂ at 25 ppm. Slight discomfort was evident when this concentration level was reached in one hour but discomfort was intolerable when exposure to the same concentration was abrupt.

In the second set of experiments it appears that prior exposures to SO₂ at low concentration has little, if any, influence on subsequent exposures at higher concentrations and that exposure concentration and the time for reaching the exposure concentration are the two factors determining the degree of response, with exposure duration having little influence once the maximum response is observed. This indicates that in situations where exposure concentration varies widely over a long time period, the use of an "average" concentration would be inappropriate, at least when considering the response of the nerve endings located in the upper respiratory tract. Therefore, this fact must be taken into account in attempting to relate the degree of response observed under various exposure (varying concentrations) conditions.

The animal model developed in mice to evaluate sensory irritation of the upper respiratory tract correlates very well with the observations made in humans and results presented above on SO₂ also indicate that the type of response that will occur with repeated exposures can be predicted.

Dr. Leland Smith (University of Texas Medical School) has also studied air pollutant-membrane interactions in an effort to discover specific molecular effects of oxidizing agents, including singlet molecular oxygen, on lipid components. He has demonstrated definitively that cholesterol is oxidized by ground-state oxygen to cholesterol 7 α - and 7 β -hydroperoxides and by excited-state (singlet) oxygen to 3 β -hydroxy-5 α -cholest-6-ene-5-hydroperoxide. These results provide means of differentiation between ground- and excited-state oxygen attack on cholesterol. These concepts have been used to exclude singlet oxygen as the oxidant in several enzyme reactions.

Dr. Smith has established that another type of secondary oxidation product, the cholesterol-5,6-epoxides, is formed by attack on initially-formed sterol hydroperoxides on residual cholesterol. Cholesterol 7-hydroperoxides formed by radiation-induced oxidation or the sterol 5 α -hydroperoxide formed by oxidation by singlet oxygen (possibly from polluted air) both convert cholesterol into cholesterol 5 α ,6 α - and 5 β ,6 β -epoxides in 1:10 ratios. The 5 α ,6 α -epoxide is a recognized carcinogen. This demonstration of the reaction pathway for its formation is of utmost importance, linking the possibilities of air pollutants with chemical carcinogenesis by this indirect manner.

Because the cholesterol 5,6-epoxides are also formed by attack of hydrogen peroxide on cholesterol, this model reaction was also examined. Hydrogen peroxide yields the same 5,6-epoxides mixture, but thermal disproportionation of hydrogen peroxide (yielding oxygen and water) also leads to cholesterol oxidation. The oxygen generated yields the cholesterol 7-hydroperoxides which in turn decompose to secondary products and isomerize to 3 β -hydroxy-5 α -cholest-6-ene-5-hydroperoxide, nominally the product of singlet oxygen attack on cholesterol. The 5-hydroperoxide also decomposes to 5 α -cholest-6-ene-3 β ,5-diol and cholesta-4,6-dien-3 β -ol, making the reaction mixtures yet more complex.

Dr. Smith has attempted without success to oxidize cholesterol with superoxide radical anion generated by passage of direct current through oxygenated electrolytes, by photosensitized reduction of oxygen, and by potassium superoxide solution. Present data do not support derivation of singlet oxygen from superoxide.

A variety of human tissues taken at autopsy were examined for oxidation products. Thus far, only the usual 7-hydroxycholesterol and 7-ketcholesterol products have been found. Chromatographic methods repeatedly support evidence of these oxidation products in tissues, but occasionally products attributed to singlet molecular oxygen reactions are also detected. Efforts are being made to refine methods so as to increase sensitivity and effectiveness of detection.

Dr. Milos Chvapil (University of Arizona) has conducted extensive studies on the stabilization and protection of membranes against toxic metals and gases. He is evaluating the effect of increased zinc levels in body fluids and tissues as a base for use as a protective or therapeutic agent. He finds that most of the Zn present in a certain membrane is linked to a definite macromolecular species constituent with a MW between 120,000 and 150,000 daltons. On

subjecting isolated and purified membrane structures to in vitro deterioration (UV radiation, lipid peroxidation by induction of NADPH oxidation or by a free radical forming system), zinc does not leak out of the membrane although several other constituents are released. This indicates that the moiety containing zinc is stable against deterioration. These conclusions are based on experiments with RBCs (red blood cells), RBC ghosts, and macrophage plasma membranes.

Dr. Chvapil has evidence that zinc stabilization of membranes is accomplished by interference in the activities of some enzymes associated with membrane deterioration. After a single i.p. injection of zinc to rats an increase in zinc plasma levels is followed by a striking accumulation of zinc in liver tissues and a concomitant decrease in NADPH oxidase and lipid peroxidase activities.

Studies on the effect of peroral zinc administration on zinc content in plasma and some organs in animals of various ages revealed that in adult or old rats or guinea pigs, it is almost impossible to induce zinc deficiency or to enhance significantly the levels of zinc in the plasma or liver tissue. Young or young adult rats reacted promptly to a high zinc diet (2,000 ppm) by strikingly enhanced zinc in various tissues. It seems that the capacity to absorb dietary zinc changes during aging. This clinically important finding is under detailed study.

Hydrocarbons

Benzene. Dr. Robert Snyder (Thomas Jefferson University) has continued studies on the biochemical events that lead to functional and morphological changes characteristic of benzene toxicity and on strategies to prevent or alleviate toxicity. He has investigated the effect of toluene on benzene metabolism and benzene induced myelotoxicity using the erythrocyte ^{59}Fe uptake method described last year. Mice were treated with either benzene alone or with mixtures of benzene plus toluene. Using radioactive labeled benzene it was found that within 24 hours about 30 percent of the administered dose of benzene was excreted in the urine and 40 percent appeared in the exhaled air. Coadministration of toluene (1720 mg/kg) with benzene (880 mg/kg) resulted in no change in amount of benzene in the exhaled air but did reduce the urinary excretion of 8 percent of the administered dose. When studied in vitro toluene, like benzene, yielded a type I binding spectrum (typical of substrates for the mixed function oxidase) when it reacted with cytochrome P-450. Toluene was also a competitive inhibitor of benzene metabolism in liver microsomes. Thus the decrease in excretion of radioactivity in the whole mouse treated with benzene and toluene appeared to be due to the inhibition of the metabolism of benzene by toluene.

Dr. Snyder previously reported that benzene inhibited ^{59}Fe uptake into circulating red cells in the mouse. However, he found that toluene has no effect on iron uptake. When toluene (1720 mg/kg) was given with benzene (880 mg/kg) the depression in Fe uptake was alleviated and was not observed when given with a lower dose of benzene (440 mg/kg). Thus, inhibition of benzene metabolism by toluene coincided with protection by toluene against the decrease in ^{59}Fe uptake into red cells, which is considered a measure of benzene toxicity.

Benzene metabolism in vivo and ^{59}Fe uptake into red cells were studied in mice that had been given either benzene alone or benzene with phenobarbital. In mice given phenobarbital (75 mg/kg for 4 days), benzene metabolism was increased by 50 percent. Fe uptake was not different in the two groups. Phenobarbital had no effect on Fe uptake in the absence of benzene. Within each group, several animals exhibited Fe uptake below 10 percent and several were above that figure. When the data were analyzed, the animals were regrouped based on Fe uptake. In the benzene group, 7 out of 22 had lower than 10 percent Fe uptake and metabolized significantly more benzene. In each case, it was observed that benzene toxicity was most severe in animals which metabolized benzene rapidly.

In an attempt to uncover a pharmacogenetic basis for differences in the rate of benzene metabolism and benzene toxicity between individual mice, 26 mice were housed in individual cages and given ^3H -benzene (880 mg/kg) once a week for 4 weeks. Urine was collected and the excreted radioactivity was calculated as the percent of the administered dose recovered in 24 hours. Each week animals were ranked from 1 to 26 with respect to the output of radioactivity. Although a few individual mice that metabolized benzene at a slow rate did tend to maintain their low rank through the 4 week observation period, overall week-to-week correlation was not significant. Thus, if the rate at which an individual mouse metabolizes benzene determines the hemopoietic toxicity of benzene, more random, non-pharmacogenetic factors are involved in determining the rate of benzene metabolism and toxicity. Additional factors such as the nutritional state of the mouse may play a role and will be studied.

Studies were conducted also on benzene binding. ^3H -benzene was incubated with liver microsomes. Proteins were precipitated with methanol:ether (3:1) until no further radioactivity could be extracted (6X). The precipitate was combusted and it was found that there was indeed residual radioactivity. The level of radioactivity indicated that benzene was bound at a level in the range of picomoles per milligram protein. This represents a rather low level when compared with some of the halogenated benzenes. However, halogenated benzenes produce liver toxicity while benzene does not.

PCBs. Extensive studies were conducted by Dr. James Allen on the metabolism and toxicity of PCB's and dioxins. Female rhesus monkeys given a diet containing 25 ppm of a polychlorinated biphenyl (Aroclor 1248) for two months developed lesions characteristic of PCB intoxication within six weeks. These lesions included loss of hair, acne, facial edema, and severe pruritus. In the more severely affected animals there was a decrease in hemoglobin, hematocrit, serum lipids, and the development of a relative neutrophilia. The major gross and microscopic findings in the animals that died included a generalized marked hypertrophy of the gastric mucosa, invasion of the submucosa by mucosal glandular epithelium, widespread submucosal edema, and formation of large mucin-filled cysts in the mucosal and submucosal areas of the stomach. In addition to the loss of hair and presence of extensive subcutaneous edema, the animals had numerous keratin cysts that developed within the hair follicles. Even after one year there was mild subcutaneous edema of the lips and around the eyes and a paucity of hair in the surviving animals. Repeated attempts to breed these animals have been successful in only one case.

These data and observations indicate that exposure to low levels of PCB in the diet for a short period is capable of causing extreme morbidity and mortality. Surviving animals continue to manifest effects of exposure to the PCBs even after a year.

Experiments have also been conducted on the tissue deposition and excretion of polychlorinated biphenyls in nonhuman primates. Juvenile monkeys were given 1.5 and 3.0 g of PCB (Aroclor 1248) per kg body weight orally. By the 14th day 5.6 percent of the original dose had been eliminated in the urine and feces by the 3.0 g animals and 5.75 percent in the 1.5 g animals. The chromatograms of the minute amounts of PCB in urine were decidedly different from those of the standard. The chromatogram of the feces included many isomers; however, many peaks of low retention time were absent or decreased in percentage.

These data indicate that a large percentage of the PCBs is absorbed from the gastrointestinal tract and subsequently eliminated slowly from the body. Likely as a result of selective absorption of the various isomers of PCB by the intestinal tract or as a result of their metabolism, many of the chromatographic peaks are inapparent or modified in the PCBs collected from the various tissues and body excreta. Indications are that specific locations of the PCBs within the various organs and tissues of the body as well as their rate of excretion may be modified by the nutritional status of the animal. In those animals that were killed after three days, the PCBs were fairly uniformly distributed in the organs evaluated. However, in the animals that were killed after two weeks, tissues such as the brain and kidney were much lower and the liver considerably higher in PCB content than similar tissues obtained from the animals sacrificed at four days. Since the fat stores throughout the body are major sites for localization of PCBs, an explanation for this relocation would be as a result of the mobilization of the PCB containing lipid material following the reduced food intake of animals that survived for 14 days.

A study similar to that reported above has been completed with a single PCB isomer 2,2,5,5-tetrachlorobiphenyl. Within 14 days approximately 10 percent of the total dose had been eliminated primarily through the feces. The greatest percentage of the absorbed PCBs was deposited in the adipose tissue and to a lesser extent in the liver and brain.

Similar experiments were conducted on chlorinated dibenzo-p-dioxins to determine their rate of excretion, deposition and effect on various organelles and tissues. In order to evaluate the rate of excretion and tissue deposition S^{35} -thioheptachlorodibenzo-p-dioxins were administered daily for 21 days. A portion of the animals was sacrificed while the remaining animals were allowed to survive for an additional six weeks. Ninety-three percent of the total radioactive dose over the 21 day period was recovered from the feces. After the discontinuation of the radioactive dioxin in the diet, all radioactivity in the feces disappeared within four days. The excretion of radioactive dioxins in the urine was quite constant throughout the 21 day period and accounted for 5.2 percent of the ingested dose. Over the following six weeks urinary excretion levels remained constant for four weeks and decreased thereafter. The amount excreted during the recovery

period amounted to 0.78 percent of the total dose. Radioactivity in the tissues was greatest in the liver, accounting for 50 percent of that present in the body. After six weeks tissue levels in the liver, adipose tissue and skin were approximately 25 percent that found after 21 days exposure to the dioxins. Analysis of the subcellular fractions of the liver revealed that 90 percent of the radioactivity of the liver was located in the microsomal fraction.

In another study, a single dose of 1, 20, and 40 ug/kg body weight of tetrachlorodibenzo-p-dioxin was given to rats, and they were subsequently evaluated for three weeks. At the doses employed, there was inhibition of growth in all animals. No weight gain was experienced by the 40 ug group while the 10 and 20 ug groups gained approximately half as much as did the controls.

The hypertrophied state of the liver cell was further substantiated by the relative decrease in DNA per gram of liver and the modifications that occurred in the microsomal fraction. The increase in microsomal protein and decrease in microsomal RNA are related to the decided proliferation of smooth endoplasmic reticulum. There was also a decided increase in the enzyme activity of N-demethylase and nitroreductase.

Alkane and alkene chlorides. Dr. Sheldon Murphy, et al. (Harvard University) have continued studies on the hepatotoxicity and lethality of 1,1-dichloroethylene (DCE) in rats. To determine if the action of DCE represented a direct hepatotoxic effect, the studies were conducted on isolated perfused livers. It was found that rats fasted overnight had significantly lower liver GSH levels than fed rats and that inhalation exposure to DCE further reduced liver GSH in both fed and fasted animals. Further experiments showed that administration of diethylmaleate to fed animals reduced their liver glutathione content by about 50 percent and markedly potentiated the hepatotoxicity of inhaled DCE. Furthermore, isolated perfused livers from fed diethylmaleate-treated rats were also more sensitive to DCE in the gas-phase of the perfusion system than were livers from fed-untreated rats.

Studies of the influence of fasting on the toxicity of several other industrial chemicals is continuing. The toxicity of 2-chlorobutadiene is the only other compound that has thus far been shown to be markedly enhanced by fasting. Fasting did not appreciably alter the inhalation toxicity of trichloroethylene or tetrachloroethylene. The chemical and toxicological characteristics of compounds that determine whether fasting or glutathione depletion alters their toxicity will be the subject of continuing studies.

Dioxins. Dr. Alan Poland (University of Rochester) has continued studies on various aspects of the toxicity of chlorinated dibenzo-p-dioxins (TCDD). TCDD was shown to be a potent inducer of hepatic δ -aminolevulinic acid synthetase activity in the chick embryo and the likely cause of porphyria cutanea tarda observed in workers in a herbicide factory. Many drugs that induce ALA-synthetase also induce hepatic microsomal monooxygenase activities, the cytochrome P-450-mediated enzymes which metabolize many foreign compounds to more polar metabolites. Preliminary studies revealed TCDD selectively

increased aryl hydrocarbon hydroxylase activity, a monooxygenase associated with a spectrally distinct active enzyme site, cytochrome P₁-450.

Fifteen congeners of TCDD were studied in the chick for their ability to induce aryl hydrocarbon hydroxylase and ALA-synthetase activities. Three generalizations emerged: (1) those congeners that induced hepatic hydroxylase activity also induced ALA synthetase; (2) the structure activity relationship among the analogues to induce these enzymes corresponded exactly with the toxicity of the compounds (lethal potency, chloracne, teratogenic potency, and capacity to produce chick edema); and (3) the structure necessary for enzyme induction was halogens in at least three of the four lateral positions (2,3,7 and 8) and a least one free halogenated carbon atom. These structure-activity relationships have been extended to over 35 dioxins and 20 dibenzofurans with similar results. The order of potency of the electron withdrawing substitutes is: Br > Cl > F > NO₂.

A comparison of the effects of TCDD and 3-methylcholanthrene, the most commonly used polycyclic hydrocarbon inducer, showed that both drugs induced hepatic aryl hydrocarbon hydroxylase activity in the rat, with parallel dose-response curves; they evoked the same maximum response; and simultaneous administration of the two drugs at maximally effective doses produced no greater induction than that elicited by either drug alone. TCDD was 30,000 times as potent as 3-methylcholanthrene. A comparative study of the time course of the events produced by both drugs showed that although maximal induction of aryl hydrocarbon hydroxylase and cytochrome P₁-450 occurred 48 hours after administration of TCDD or 3-methylcholanthrene, induction persisted over a month after a single dose of TCDD whereas the response evoked by 3-methylcholanthrene returned to normal in a week. TCDD was superior to 3-methylcholanthrene in inducing aryl hydrocarbon hydroxylase activity in extrahepatic tissues. A comparison of the log-dose response curves of induction of hepatic aryl hydroxylase activity by TCDD in chick embryos, Sprague Dawley rats, and inbred strains of mice showed that the dose eliciting half the maximal response (ED₅₀) was quite similar, ranging from 0.4 to 1.2 x 10⁻⁹ moles/kg. In summary, TCDD is similar to 3-methylcholanthrene in its effects on microsomal monooxygenase, differing primarily in its greater potency and duration of action. The prolonged duration of action of TCDD is presumably related to its long half-life and resistance to metabolism.

The extraordinary potency of TCDD prompted examination of the expression of aryl hydrocarbon hydroxylase in inbred strains of mice which fail to respond to the usual aromatic hydrocarbons, such as 3-methylcholanthrene or β -naphthoflavone. Nebert and coworkers have shown that in some inbred strains of mice, so-called genetically "responsive" (to aromatic hydrocarbons), the administration of 3-methylcholanthrene induces aryl hydrocarbon hydroxylase activity, three other microsomal monooxygenase activities and the formation of cytochrome P₁-450. However, 3-methylcholanthrene administration to other, genetically "nonresponsive" inbred strains elicits none of these changes. In the prototype cross, C57Bl/6 (responsive) mice x DBA/2 (nonresponsive) mice, the locus that controls aromatic hydrocarbon responsiveness segregates as a simple autosomal dominant.

In both responsive and nonresponsive mice, the administration of TCDD induces aryl hydrocarbon hydroxylase activity, the three other linked microsomal monooxygenase activities and cytochrome P₁-450. This demonstrates that the nonresponsive mice have the regulatory and structural genes necessary for the expression of aryl hydrocarbon hydroxylase activity and the formation of cytochrome P₁-450. The defect in these mice seems to be the failure to recognize the usual aromatic hydrocarbons. In biochemical terms, this suggests a mutation that results in a defective induction receptor with diminished binding affinity for aromatic hydrocarbon compared to genetically responsive mice.

Among approximately twenty inbred strains of mice, the so-called genetically "nonresponsive" strains are less sensitive to TCDD than the "responsive" strains, and the dose-response curves of the nonresponsive strains are shifted to the right. The ED₅₀ of hepatic hydroxylase induction by TCDD is 1×10^{-9} moles/kg in responsive strains and 1×10^{-8} moles/kg in nonresponsive mice. The heterozygous hybrids of responsive and nonresponsive parents are intermediate in their sensitivity. Furthermore, administration of a low dose of ¹⁴C-TCDD (114 mCi/mole), 6×10^{-9} moles/kg, resulted in a high hepatic uptake in responsive strains, a low uptake in nonresponsive strains and an intermediate uptake in heterozygous hybrids.

Chemical Mechanisms

Aromatic Degradations. Dr. Stanley Dagley (University of Minnesota) has continued work to delineate the pathways of microbial catabolism of benzenoid compounds and to isolate, purify and characterize the enzymes involved. He has isolated the intermediates in the degradation of 4-hydroxyphenylacetic acid (I) by *Pseudomonas putida* and *Acinetobacter* and has purified some of the enzymes involved in degradation. One (4-hydroxyketopimelate aldolase) has MW = 158,000. It is globular and consists of six apparently identical subunits of MW = 25,700. It is stimulated by Mg⁺⁺ and Mn⁺⁺ although an absolute requirement was not demonstrated. This description almost exactly fits 4-hydroxy-4-methyl-2-ketoglutarate aldolase, purified in this laboratory in 1972; however, the two aldolases show narrow substrate specificities that do not overlap.

The properties of 4-hydroxyphenylacetate 1-hydroxylase, which converts I to homogentisate with migration of the side-chain substituent during hydroxylation of the benzene nucleus was also described. FAD is required while NADH or NADPH (less effective) are oxidized when O₂ is consumed during hydroxylation. A requirement for Mg⁺⁺ ions is shown which is an unusual feature for a hydroxylase.

Nineteen analogues of I were tested as substrates or inhibitors. Strong inhibition, competitive with I in each case, was exhibited by three compounds; and of six compounds showing some substrate activity, two were attacked at about half the rate of compound I, namely 4-hydroxyphenoxyacetic acid and 4-hydroxyhydratropic acid. A mandatory structural feature for activity, either as effective inhibitor or as substrate, was the presence of a hydroxyl group at C-4. This suggests a somewhat different mechanism from that of NIH shifts studied for microsomes. Dr. Dagley proposes a mechanism

involving a carbonium ion intermediate and quinonoid forms. This is strongly supported by the observation that the enzyme does not catalyze a migration in the case of 4-hydroxyphenoxyacetic acid, but instead detaches the side chain as glycollate leaving benzoquinone, which is immediately reduced to hydroquinone by another enzyme. Moreover, "migration" of the side chain of I appears to be a misleading description since one enantiomer only of 4-hydroxyhydratropic acid is attacked, and one (optically active) enantiomer of C α -methyl-substituted homogentisate is given. It appears from this and other evidence that the side chain is bound to a site on the enzyme throughout the course of the reaction. Presumably it is the nucleus which moves the short distance to enable a transfer of the side chain from C-1 to C-2. This could occur through a conformational change in the enzyme during the reaction.

A third enzyme, maleylpyruvate hydrolase, has been purified from a pseudomonad that degrades gentisate to give maleate and pyruvate. The enzyme has MW = 62,000 with two monidentical subunits each of MW = 31,000. On electrophoresis, two very close bands are given both of which possess catalytic activity, as shown by taking thin slices of a polyacrylamide gel. The substrate specificity of the hydrolase has been examined for a wide range of analogues, including several compounds containing halogens.

Related studies on enzymatic oxygen fixation into aromatic hydrocarbons have been carried out by Dr. David Gibson (University of Texas, Austin). He has developed a procedure for preparation of extracts of *P. putida* that oxidize toluene. Apparently at least two proteins are required for toluene dioxygenase activity. These have not yet been purified. Dr. Gibson has shown also that cells of *P. putida* contain an enzyme that fixes molecular oxygen into catechol with the formation of 2-hydroxymuconic semialdehyde. This enzyme was found to be unstable. However, the presence of 10 percent acetone in crude cell extracts preserved activity for a 72 hour period. The enzyme was purified to homogeneity. The final preparation showed a 37-fold purification with a recovery of 5 percent. The enzyme gave a single band when stained for protein on disc gel electrophoresis that was associated with enzymic activity. When electrophoresis gels were suspended in 0.02M KH₂PO₄ buffer, pH 7.5, containing 1.0 mM Catechol, a bright yellow band appeared on the gel that corresponded in mobility to the band stained for protein. The yellow band indicates the formation of 2-hydroxymuconic semialdehyde.

A mutant strain of a *Biejerinckia* species was found which oxidizes phenanthrene and anthracene. The oxygenated products were isolated and identified as (+)-*cis*-3,4-dihydroxy-3,4-dihydrophenanthrene and (+)-*cis*-1,2-dihydroxy-1,2-dihydroanthracene, respectively. Structure assignments were deduced from the information provided by ultraviolet and infrared spectrometry, acid catalyzed dehydration of the metabolites to phenols which were isolated and identified and the formation of isopropylidene derivatives with 2,2-dimethoxypropane.

Physical Factors

Taste. Dr. Linda Bartoshuk (John B. Pierce Foundation) has attempted to evaluate the health implications of unpleasant tastes in drinking water. She has found that the taste of water per se depends primarily on the oral environment, particularly on saliva. Salivary constituents can vary widely. In particular, some diseases (e.g., cystic fibrosis, Addison's disease, hypertension) elevate Na and Cl concentrations. This source of variability in the taste of drinking water has not previously been examined.

Mixture suppression has turned out to be an extremely important variable in the study of taste quality. In the laboratory, most taste stimuli are chosen for their ability to produce pure tastes. In the environment, most taste stimuli are complex. Determining the laws that relate simple and complex tastes will not only permit generalization of the principles determined with simple stimuli but will also permit the development of powerful techniques for the control of taste quality. Interactions among tastes in a mixture provide the main technique currently used to alter taste quality and thereby improve palatability. Determining the laws underlying what has been empirical technique would permit choosing the safest, cheapest components that could produce the desired result. This would have application to the improvement of food palatability as well as to that of drinking water.

In other studies, Dr. Bartoshuk has attempted to determine NaCl thresholds in children with cystic fibrosis (CF). It was previously believed that CF children have taste thresholds markedly lower than normal. However, the results of these experiments indicate that when saliva was removed by rinsing prior to the threshold tests, the NaCl thresholds of the normal and CF children were the same. CF children have higher concentrations of electrolytes than normal children. Adaptation to these electrolytes may have caused the water diluent in previous experiments to taste bitter. This could have produced low thresholds that were actually thresholds for water rather than NaCl taste.

The basis of sweet water tastes induced by potassium chlorogenate (an artichoke constituent) and potassium chlorate was also investigated. Responses to water following various concentrations of K chlorogenate were obtained from 40 subjects. In the same session, another salt known to induce a sweet water taste, K chlorate, was similarly tested. Although most subjects noted some sweet taste, only certain subjects noted a sweet taste that increased in intensity as the adaptation concentration of the salt increased. In all cases, sensitivity to K chlorogenate is very expensive while K chlorate is relatively cheap. Subsequent research on the mechanism can now be done primarily on the cheaper K chlorate.

Other experiments on cats show that the taste evoked by water in the normal cat adapted to its own saliva can obscure the taste of sucrose. Since this water taste could also potentially obscure the tastes of nonnutritive sweeteners to the cat, existing data on cat preference for sweeteners seems of doubtful accuracy. Saccharin, Ca cyclamate and dulcin were tested by providing cats with a choice between the sweetener dissolved in .03 M NaCl and

.03 M NaCl alone. This concentration of NaCl prevents the occurrence of the taste normally evoked by water consumed when the tongue is adapted to the NaCl in saliva. Cats rejected saccharin and Ca cyclamate but were indifferent to dulcin. Rejection of saccharin occurred at much lower concentrations of saccharin than previously reported, suggesting that the taste of water may well have partially obscured the sweetener taste in previous studies.

At the same Institution, Dr. William Cain has conducted various experiments directed at issues in odor pollution. Experiments performed with subjects who possess normal olfactory and trigeminal sensitivity in one nostril and normal olfactory, but no trigeminal sensitivity in the contralateral nostril show that the trigeminal nerve contributes substantially to the overall perceived magnitude of high intensity stimuli. At high levels, the course of adaptation was more rapid in the nostril possessing no trigeminal sensitivity than in the normal nostril. At low levels of stimulation the trigeminal nerve had little or no influence on the course of adaptation.

In studies on odor masking and counteraction, it was found that (a) a mixture of two odorants does not smell as strong as the sum of the components smelled separately; (b) when one component in a binary mixture is 3 to five times as strong as the other component, the mixture will smell less intense than the stronger component, exemplifying odor counteraction; (c) any odorous substance can counteract the odor of any other substance, since odor counteraction merely reflects how the olfactory system processes those mixtures where the components are present in unequal proportions; (d) the ability to mask the presence of a malodor varies from substance to substance and seems to depend on how "rich" is the quality of the masking agent; (e) a model employing vector addition provides an excellent description of how the components of a mixture combine to produce the overall odor intensity of the mixture. The stimuli used in these experiments include pyridine, propanol, amyl butyrate, linalool, linalyl acetate, and lavandin oil.

Pathogenesis

Pesticides - Behavior

The effects of DDT and parathion on the behavior of the rat were investigated by Drs. Albert Casey and Anthony Bourgeois (Texas A & M University). Behavioral parameters investigated were motivation, emotion, learning, activity, and certain effects potentiated by cold stress. Intraperitoneal injections of the compounds at 10 and 25 percent of the LD₅₀ doses did not have a statistically significant effect on motivation, emotionality, acquisition of a position task in a Y wading maze, extinction of a conditional avoidance response, passive avoidance tasks, stimulus-induced activity or general activity. There was a slight effect produced by parathion on reversal of a previously acquired response on the first and fifth post-injection days.

A secondary purpose of these investigations was to develop a model of critical behavioral functions and their related measurements and associated experimental techniques that would serve the broader field of behavioral toxicology. Progress was made in this respect with the tailoring and refinement of procedures for studying toxic effects, e.g., the training procedures for delayed performance, measures of resistance to fatigue, etc.

A general finding was that at the times measured, behavior is relatively resistant to the types of agents and doses employed. This was true of measures of motivation, emotion, discriminative avoidance, passive avoidance, learning to reverse a position habit in a water maze, acquiring a position habit in a Yerkes discrimination apparatus, general activity, and cold potentiation of DDT. However, there were exceptions. The learning of a position habit in a water maze was retarded on the first day following injection of a 25 percent LD₅₀ dose of DDT and on the third day following a 25 percent LD₅₀ dose of parathion. In the Yerkes discrimination apparatus, a 35 percent LD₅₀ dose of parathion significantly retarded the reversal of an established position habit on the first and fifth days following injection.

NO₂ and ozone - Lung disease

Dr. Gustave Freeman, et al. have continued studies on chronic obstructive lung disease caused by oxides of nitrogen and ozone individually and together. During the past year, rats that had been exposed intermittently for a lifetime (about 2 1/2 years) to about 15 ppm NO₂ developed emphysema, a chronic obstructive lung disease (COLD) and exhibited an attendant loss of oxygen-absorbing surface area in the lung. This resulted from destruction of tissue, with a loss of about two-thirds of the air sacs (alveoli). Stagnant air was retained in the enlarged, obstructed lungs following their removal from the chest. The main impact of injury initially was in the smaller airways that conduct gases immediately to and from the alveoli. These features resemble those in man and constitute a reasonable facsimile of human emphysema.

Ozone has proven to be approximately twenty times more injurious than NO₂ to the lungs of rats. It induces a similar type of disease but with certain significant differences. These are, mainly, a slight deeper (more distal) position in the lungs of the initial injury and an early stimulation of connective tissue growth at the sites of injury. Considerable reaction becomes apparent within a few hours of exposure to less than 0.5 ppm--a concentration readily achieved at peak traffic hours in some urban areas. Cellular changes are obvious within four hours of exposure to about 0.9 ppm O₃, a level occasionally reached in heavy smog. Thus, it is important to investigate the combination of smoking and living in photochemical smog, because smokers inhale concentrations of oxides of nitrogen from cigarettes also.

A mixture of concentrations as low as 0.25 ppm O₃ and 2.5 ppm NO₂ caused definitive injury to the lining cells of the small airways in the lungs within 24 hours; and a mixture of each at the occasionally achieved, high ambient level of about 0.9 ppm, induced an advanced, lethal degree of emphysema within two months of continuous exposure.

Polycythemia (an excessive concentration of red cells) developed in weanling rats exposed to O₃, as it did in those treated with NO₂. When growing or

mature rats were exposed to NO₂, their red blood cells were reduced in size, although their numbers and durability increased to above normal. In contrast, rats exposed from birth to about 15 ppm NO₂ had an absolute as well as a relative reduction in the number of red cells compared with unexposed rats of the same age. Also, their cells tended to be larger than normal. Simultaneously, the amounts of ATP were reduced.

When the airways and air sacs were naturally exposed to as little as 2 ppm NO₂ during respiration, the usual rate and replacement of the epithelial (surface) cells was accelerated, reaching a maximum during the second and third days and returning to the pre-exposure rate within a week of continuous exposure. Accelerated division of certain cells (Type 2) of the alveolar walls and of nonciliated cells lining the walls of the small airways (bronchiole) occurred to replace the injured cells. This was determined quantitatively by periodically counting the various types of cells showing radioactive evidence of their preparation to divide. Light and electron microscopy revealed the sequence of events starting a couple of hours after the initiation of exposure to either 15 ppm NO₂ or to 0.5 ppm O₃ and depicted sloughing of ciliated cells from the small airways and of the thin, flat Type 1 cells from the adjoining alveolar walls. However, following short-term exposure and allowing time for replacement of the dead cells, the new cells that appeared were relatively resistant to the same concentrations of gases.

In view of the striking reactivity that occurred between pure vitamin E and NO₂, a biochemical method was developed to determine the amounts of vitamin E present in tissues such as the lungs. This difficult procedure is now being used in experiments comparing the organs of NO₂-exposed animals with those of unexposed animals.

Changes in both ATP and 2,3-diphosphoglycerate (2,3-DPG) in the red blood cells were correlated tentatively with the numbers of cells and their altering characteristics during maturation in an environment containing NO₂. Newborn rats develop anemia, whereas rats newly exposed at a month of age or older develop polycythemia. In parallel, the cells change in size from overly large in newborn rats to smaller than normal in comparison with red cells in unexposed animals of the same ages.

Dr. Jean Acton (Bowman Gray) has studied NO₂-induced alterations in lung macrophage function. She found that there are no qualitative differences in total lipid, cholesterol or fatty acid composition of normal and NO₂-exposed macrophages or the surfactant-containing lavage fluids.

Pilot experiments on the effect of NO₂ on macrophage permeability indicated that measurement of lactic dehydrogenase (LDH) release from cells is a more sensitive measure of cell damage than either cell viability or osmotic sensitivity. When cultured in vitro, alveolar macrophages washed from the lungs of animals exposed to 25 ppm of nitrogen dioxide for three hours released significantly more LDH into the supernatant fluid than macrophages from normal rabbits. Since small amounts of enzyme appear in the supernatant fluid of control cell cultures, it is apparent that some cell damage and leakage occurs because of the culturing process. If cells from animals exposed to NO₂ synthesized more enzyme than normal cells, the increased levels

in the supernatant fluid might not reflect NO₂ induced membrane damage. Experiments were designed to measure intracellular and extracellular LDH concentrations so that changes in surface membrane permeability could be calculated. Although there was considerable animal to animal variation, both supernatant and total LDH activities were significantly higher in NO₂ exposed cells than in control cells. Thus, it is apparent that the permeability of the NO₂ exposed macrophages increased by 70 to 125 percent over the control value. Even if for the one hour total enzyme samples, the upper standard value is used for the NO₂ and the lower standard deviation value for the control, there is a 40 percent increase in permeability.

Dr. Daniel Menzel (Duke University) has continued studies predicated on the concept that NO₂ and O₃ may initiate lipid peroxidation, which might account for lung pathology. He has studied the role that vitamin E might play as a membrane lipid antioxidant in retarding such effects. In prior work, it was noted that the products of the ozonolysis of polyunsaturated fatty acids were not always equivalent to the peroxidation hypothesis, e.g., oxidized compounds other than hydroperoxides or hydroperoxide decomposition products were detected. Fatty acid ozonides were found to be the principal products under anhydrous conditions. Subsequently, it was found that much of the gross pathology of ozone inhalation could be duplicated by injection of methyl ozonides of fatty acids. Methyl ozonides and free fatty acid ozonides were also found to initiate lipid peroxidation of isolated organelles.

The experiments to date support the original hypothesis that ozone and nitrogen dioxide affect their toxic action via lipid peroxidation. Intermediary chemical species (fatty acid ozonides) appear to be the ultimate toxicant. These compounds can be formed in serum and produce a gross pathology similar to ozone inhalation. The ability of fatty acid ozonides to initiate lipid peroxidation as well as oxidations of cytosol thiols and polymerization of soluble proteins suggests that they may form the branch point consolidating the previous observations of both lipid peroxidation and thiol oxidation during ozone inhalation. These two hypotheses are not, therefore, mutually exclusive and may explain why both lipid antioxidants (vitamin E) and thiol generating systems are effective in protection against ozone inhalation. The observations with human erythrocytes suggests that the animal models are similar to man and that prevention by suitable compounds is likely. These observations on the applicability to man are encouraging in light of the present trend toward greater air pollution levels as a result of the energy crisis.

Dr. Jerome Kleinerman (Saint Luke's Hospital) has conducted extensive studies on the mechanisms of experimental emphysema. In experiments with NO₂ and dust alone and in combination, he finds that the upstream resistance is significantly increased in animals exposed to NO₂ as compared to those exposed to dust alone or in controls. No significant differences are observed between controls and dust/NO₂ at higher lung volumes. However, as lung volume decreases, the difference between dust/NO₂ and control values become significant. No significant differences are observed between dust/NO₂ and NO₂ groups. The effect on upstream resistance which reflects the changes in the resistance in the peripheral airways is significantly increased by NO₂ exposure with or without dust.

Dust foci were observed on the surface and in the parenchyma of dust exposed animals. These pigmented foci were recognized on histologic section as collections of pigment-filled macrophages within alveolar ducts or respiratory bronchioles. Few were in interstitial tissue. No fibrosis or emphysema was observed. Respiratory bronchiolitis was not present and only a few hyperplastic epithelial foci in the respiratory bronchioles were noted. No evidence of emphysema, fibrosis or neoplasia was observed.

A new study was initiated to evaluate the effects of an additional respiratory epithelial irritant superimposed upon those previously studied. Diethylnitrosamine (DEN) is a substance reported to produce epithelial neoplasms in the respiratory, gastrointestinal and urinary tract of other mammals. It is unique in that its effects are observed long after the administration of the drug. The hypothesis which underlies this study is that DEN superimposed upon previously injured respiratory tract epithelium will augment a carcinogenic stimulus and produce lesions at an earlier time than that expected from the drug administration alone. An extremely small dose of DEN was used in order to evaluate the potentiating effects of the superimposed injury of NO₂ and dust. Animals were exposed to NO₂ in concentrations of approximately 20 ppm for 20 to 22 hours daily, seven days per week. Hamsters were exposed to fly ash collected in the precipitators of the Cleveland Electric Illuminating Company in concentrations of approximately 4 mg/cubic meter. DEN was injected subcutaneously once a week in doses of 1 mg per injection for seven consecutive weeks. As of August 31, 1974, no evidence of tumor formation had been observed in those animals which died.

In other studies the effects of intravenous elastase in the production of emphysema were determined. This study was based on the concept that in man proteolytic enzymes including elastase exist within the neutrophil. If elastase is to be effective in producing emphysema in man, the leukocytic elastase must find its way from within the vascular bed to the site of elastic fibers in the pulmonary parenchyma. This requires passage of leukocytes or leukocytic enzymes transendothelially into pulmonary circulation. Animals exposed to elastase exhibited localized areas of panlobular emphysema. The limited extent of the lesion is believed to be the result of the limited dose of elastase which reaches the lung parenchyma and the extensive reservoir of intravascular serum antiproteases available to inactivate the elastase. The absence of observable pressure volume changes is not unusual when the amounts of emphysema present are limited to less than 15 to 20 percent as is the case in this study. These studies suggest that proteolytic enzymes as exemplified by elastase may produce emphysema when delivered via the intravascular route.

In similar studies with papain, Dr. Kleinerman has tested the effects of agents that produce leukopenia, e.g., cytoxan (CTX) and 5-fluorouracil (5-FU). From previous work with papain it was postulated that papain may act upon circulating plasma proteins to produce a chemotactic factor in the lung which would cause a leukocytic influx into the lung. These leukocytes were postulated to produce lung injury by release of lysosomal proteases. The studies this year indicate that papain-induced emphysema is not diminished by the leukopenia induced by CTX. This suggests that the mechanism of action of papain, and possibly other proteolytic enzymes, in producing experimental

emphysema is very likely by direct action on the lung parenchyma and elastin and not via action upon the host circulating leukocytes.

Carrageenan. Dr. R. Abraham (Albany Medical College) has attempted to elucidate the mechanisms of the ulcerogenic response to guinea pigs to degraded carrageenan (C¹⁶). In a comparative study of rhesus monkeys, rats and guinea pigs receiving various concentrations of C¹⁶ it was noted that ulceration occurred only in the ceca of guinea pigs. A significant feature in the ulcerative process was the ability of the macrophages in the lamina propria of the guinea pig to endocytose and store C¹⁶, which brought about stimulation and release of lysosomal enzymes with damage to surrounding tissue. Cecal ulceration was not seen in the rhesus monkeys or rats, even where the macromolecular material was not trapped in the macrophages of the lamina propria.

In studying the dietary effects influencing carrageenan-induced ulceration in guinea pigs, a notable finding was that the protective effects were afforded by milk and diet in the ulcerative process. When comparable levels of C¹⁶ were either dissolved in milk or given in the diet to guinea pigs, no ulceration was seen, but instead, pigment (probably derived from protein in milk) was present in macrophage lysosomes that originally had C¹⁶.

An important aspect in the safety evaluation of various carrageenans used as food additives concerns the effects of high and low molecular weight materials. A series of experiments in rats and guinea pigs are in process, some of which have been completed, to determine the rates of absorption, retention and histopathological results of the storage of six fractions of i-carrageenan of molecular weights 5,000; 8,700; 21,000; 39,000; 88,000; 107,000; and 145,000; three fractions of K-carrageenans (molecular weights 8,500; 51,500; and 314,000) and Λ -carrageenans (molecular weights 20,000; 74,800; and 275,000). Preliminary studies indicate lack of metachromasia and storage in the three highest fractions, the two highest K and the highest Λ fraction. The histopathological and ultrastructural effects of absorption of the lower molecular weight materials is under investigation.

Dr. Abraham has also studied the interactions of carrageenans with other chemicals. The permeability and functional state of carrageenan-laden lysosomes was tested by exposure to horseradish peroxidase, chloroquine, and iron dextran. These studies demonstrated that while carrageenan-laden lysosomes were permeable to iron-dextran, they were unable to take up horseradish peroxidase or chloroquine, a finding that has interesting implications with regard to the known digestive and therapeutic roles that lysosomes display.

He has also studied the role of lysosomes in pesticide-induced liver enlargement (dieldrin, PCBs and Mirex). The intent of these studies is to determine a no-effect level of the pesticides and to study the mechanism involved in liver enlargement. One approach has been to observe the role of lysosomes, and in particular, whether a lack of catabolism by these organelles results in an increase in liver size. Efforts were made to stimulate synthesis of lysosomal enzymes by administration of a combination of pesticides. Preliminary results involving PCB and Mirex demonstrated that individual

compounds exert no effect of lysosomes, but that administration of two polychlorinated compounds produces striking structural changes in hepatic lysosomes with the formation of large secondary lysosomes (containing cellular organelles and cellular debris). Lysosomal changes in the liver cells of these animals were associated with a reduction of cell size (hyperplasia) rather than hypertrophy. In the context of liver enlargement, porphyria is also produced by polychlorinated biphenyls. What role the lysosomes play in the sequestration and transport of porphyrins is being investigated, initially by the use of some model compounds that produce porphyria.

Cadmium. Dr. Harvey Gonick (University of California School of Medicine) has continued studies on the pathology of the kidney in relation to Cd toxicity. An experimental Fanconi model was achieved by repetitive intraperitoneal injections of Cd++ as CdCl₂. Glycosuria and aminoaciduria appeared abruptly after approximately three weeks of injections and disappeared gradually over a four to five day period after discontinuation of injections. The complete expression of the Fanconi syndrome was elicited, i.e., diuresis, glucosuria, aminoaciduria, proteinuria and increased fractional excretion of Na, K, Ca, P and Mg. Urate excretion was marginally increased.

Animals were studied at certain specific phases; (a) control; (b) two days prior to the anticipated development of Fanconi syndrome; i.e., "pre-Fanconi" (group 0); and (c) on the first day (group I), second day (group II), and third day (group III) of development of Fanconi syndrome. Measurements of plasma and urine Cd and kidney cortex, kidney medulla and liver content of Cd and Zn were made during each of these phases. In addition, ATP levels were measured in renal cortex and enzymatic activities of Na-K-ATPase, Mg-ATPase, acid phosphatase and β -glucuronidase were measured in both cortex and medulla. Plasma Cd remained relatively unchanged throughout the experiment. Urine Cd increased only after the second day of Fanconi syndrome, while tissue Cd reached near maximum levels in the pre-Fanconi phase and increased only slightly thereafter. Both ATP levels and Na-K-ATPase activity of kidney cortex were reduced in groups I and II, correlating with the onset of Fanconi syndrome.

The ultrastructural changes in the kidney at each phase of development of the Fanconi syndrome have been evaluated. Ultrastructural changes were confined almost exclusively to the proximal tubule. The pre-Fanconi animals showed increased mitochondrial density and contraction with effacement of the crestae pattern in many but not all mitochondria. Early disruption and loss of the basal cytoplasmic membrane infolding was noted and in these areas complex arrays of delicate cisternal structures resembling proliferating smooth endoplasmic reticulum were seen. Abnormalities in the group II Fanconi animals were more advanced, consisting of dramatic alterations in size, configuration and distribution of mitochondria and extensive loss of the basal plasma membrane infoldings together with complex cisternal proliferation. Thus, the ultrastructural changes, the biochemical alterations in the Na-K-ATPase-ATP transport system and the renal tubular functional changes developed in a parallel sequence in man and animals (Cd++, Hg++, Cu++, U++ and Pb++) were found to be potent inhibitors of Na-K-ATPase *in vitro*, 50 percent inhibition occurring at concentrations varying from 10^{-4} M (U++) to 5×10^{-6} M (Hg++).

The effect of Cd on active transport by the isolated frog skin is being studied. No effect on short-circuit current (SCC) was noted when Cd was added to the serosal surface in doses of $10^{-4}M$ and $10^{-3}M$. On the other hand, when Cd was added to the mucosal surface, a significant stimulation was noted at both dose levels (33 percent and 57 percent). The stimulatory effect of Cd was additive to the effects of either vasopressin or theophyllin and thus did not appear to act via a cyclic AMP mechanism. It is postulated that the lack of inhibitory effect of Cd when added to the serosal surface is due to failure to penetrate to the site of Na-K-ATPase activity and that the stimulatory effect of Cd when added to the mucosal surface may be due to a direct interaction with the membrane.

Dr. B. Chin (University of Michigan) has studied the time and mode of action of cadmium in relation to cell cycle. Peak sensitivity to the metal ions appears to be in late G_2 phase. Exposure at that stage results in inhibition of protein but not DNA synthesis.

Similar macromolecular studies are planned for the cadmium-sensitive period in early S phase. Interest in this period is prompted by the demonstration that continuous exposure to $4 \times 10^{-4}M$ cadmium during S results in a 60 percent increase in incorporation of H^3 -TdR. These biochemical studies indicate that the nucleus is a subcellular target for cadmium.

Failure to isolate a low molecular weight, Cd^{109} -binding protein by classical preparative methods indicates that protection is not provided by a metallothionein. Experiments are in progress to develop means to determine if protection may result from an enhanced ability to exclude Cd, or to redirect it to a non-toxic site. Preliminary studies indicate that the subthreshold dose decreases retention of labeled Cd in the suprathreshold dose. This approach is being redesigned to monitor the fate of Cd from both exposures by a combination of atomic absorption and radioisotopic counting.

Action spectra for cycloheximide and Cd in the last half of the cell cycle contain coincident peaks of sensitivity. It is postulated that mitotic delay caused by exposure to Cd in late- G_2 may result from inhibition of a phase-dependent synthesis of a mitotic protein. This hypothesis predicts that prior exposure to a subthreshold dose should protect the cell against inhibition of protein synthesis by the suprathreshold dose of Cd. Initial experiments indicate this to be the case. However, the data would not exclude the inhibition of RNA synthesis as the causal event and experiments are in progress to examine this possibility.

Lead and Mercury. Dr. Werner Niklowitz (University of Cincinnati) has studied the effects of lead and mercury on components of selected areas of the central nervous system at the cellular and subcellular levels.

Emphasis has been placed upon (1) determining the cytopathological changes before and during the onset of neurological manifestations of toxic effects, (2) evaluating the content of toxic metals and any interference with essential trace metals in these brain areas, and (3) providing analytical data for better understanding of the focal subcellular mechanisms of the action of toxic metals in the central nervous system.

After exposure of rabbits to tetraethyllead (TEL), nerve cell components of selected brain areas (frontal cortex, cerebellum, and hippocampus) were studied for ultrastructural effects. A single dose of 100 mg/kg of TEL, after a latent interval of 12 to 18 hours, is followed by neurological disorders such as convulsions and produces significant alterations of components of pyramidal and astroglial cells. These ultrastructural changes develop before the onset of major neurological signs of toxicity. In general, two types of degenerative cell changes are identified: cellular sclerosis and cellular hydrops, each specific to certain cell layers of selected brain areas. The subcellular changes in nerve cells undergoing hydropic degeneration exhibit a distinct pattern. The following sequence of appearance of ultrastructural alterations was determined: (1) folding of the nuclear membrane; (2) swelling and severe lesions of mitochondria; (3) hypertrophy of Golgi complexes and (4) patchy chromatolysis of the ergastoplasm (Nissl substance). Progressive hypertrophy of Golgi saccules and vacuolization of ergastoplasm areas lead to a vacuolar hydropic degeneration of affected nerve cells.

Dimethylmercury (DMM) causes nerve cell changes similar to those after TEL poisoning. However, the progressive loss of perikaryal substances and organelles in pyramidal cells undergoing hydropic degeneration, the lack of distinct patchy intracellular lesions, as seen after TEL intoxication, and the advanced lysis of mitochondria in nerve cells sclerotic as well as hydropic degeneration indicate that DMM poisoning affects the nerve cells acutely and to a greater degree of severity than organolead (TEL).

After exposure of rabbits to TEL (100 mg/kg) each investigated brain area contained lead in concentrations of approximately 150 nmoles per gram dry weight. Preliminary data indicate that at the same time, each brain area revealed a statistically significant loss of essential tissue copper, iron and zinc. While the ratio between gain of lead and loss of copper revealed a constant proportionality of 1:1, the decrease of iron and zinc levels varied.

After exposure of rabbits to DMM in amounts equivalent to the molar concentrations of Pb used in the TEL experiments, analysis revealed that these brain areas contained approximately equimolar concentrations of Hg comparable to those of Pb after TEL intoxication. Furthermore, the investigated brain areas exhibited a shift of essential trace metals as revealed by similar decreases of Cu, Fe and Zn.

Dr. Billy Kirk (West Virginia University) has attempted to evaluate the effect of lead in the diet of mice on their ability to accept or reject tumors. It was postulated that any change in tumor induction would be a result of some change in the immune responses. Both the transplantable leukemia L1210 cell and the Moloney sarcoma virus were used during the course of the project since the immune response to a leukemia and a solid tumor appears to be different.

Preliminary studies in C3H mice indicate that feeding 5 percent lead acetate delays the time of maximum L1210 cell number approximately two days. This is similar to the results seen with Swiss mice. No difference in the time of rejection of tumor cells was noted between groups.

Studies on the induction of tumors with Moloney sarcoma virus are in progress. Virus stocks have been prepared and experimental techniques are being developed for virus assay and assay of immune response cells transformed by this virus. Experiments to determine the incidence and progression of virus-induced tumors in BALB/c mice fed varying amounts of lead acetate are in progress. Indicators to date are that the effect of lead on tumorigenesis is not dramatic but that lead in general has a depressing effect. This agrees with some of the published literature, but contradicts other reports.

Dr. Richard Manalis (University of Cincinnati) has continued studies on the effects of lead and mercury ions on the chemical synapse. It was shown that lead affects the release of transmitter from nerve endings in two different ways. First, it increases the frequency of the spontaneous or resting release of acetylcholine, the neurotransmitter, from the motor nerve terminals. Second, it concomitantly inhibits the phasic release of transmitter; that is, the amount of transmitter released from the nerve terminals following the arrival of a nerve impulse is reduced in the presence of lead. This dual action of lead clearly demonstrates that these two processes of transmitter release--the spontaneous release and the phasic release--occur via different mechanisms because the same agent, lead, excites one and inhibits the other.

Lead is about 1,000 times more potent on a molar basis than is magnesium in inhibiting the phasic release of transmitter. The blocking effect of lead is also overcome by elevating the concentration of calcium in the bathing medium.

In order to confirm that lead reduced the endplate potential through a presynaptic mechanism and not by lowering the membrane resistance of the muscle fiber, resistance measurements were made. It was found that 50 micromolar lead added to the Ringer's solution caused no significant change in the membrane resistance.

Dr. A. M. Goldberg (Johns Hopkins University) has studied the effects of lead on neuromuscular function using the phrenic nerve-diaphragm preparation of the rat. Hemidiaphragms were exposed to lead in concentrations from 1×10^{-5} to $1 \times 10^{-4}M$ and its pair run as controls. At $2.5 \times 10^{-5}M$ or greater the force of contraction produced by nerve stimulation declined significantly. The magnitude of the twitch response decreased as the concentration increased. Direct electrical stimulation of muscle produced the same response in control and treated preparations. Exogenous acetylcholine in submaximal concentrations produce equal responses in treated and untreated preparations in the absence of stimulation. The results that lead produces a specific lesion at the presynaptic level.

In order to define more accurately the transmission failure produced by lead, it was necessary to establish dose-reaction relationships. The isolated nerve-muscle preparation was exposed to concentrations of lead up to $1 \times 10^{-4}M$ and the force of contraction, either by stimulation through the nerve or by the muscle directly, was measured after two hours of exposure of lead. The results demonstrated that at $1 \times 10^{-5}M$ no defect in transmission occurred.

However, as the concentration of lead increased, the force of contraction decreased when stimulation was initiated through the nerve. At all concentrations of lead studied, there was no defect in the ability of the muscle to respond.

Since it appeared that the muscle was capable of responding to direct stimulation but that a failure occurred between nerve and muscle it was necessary to test the sensitivity of the neuro-muscular junction to exogenous acetylcholine. The hemidiaphragm preparations were exposed to $8 \times 10^{-5}M$ lead for two hours. At the end of two hours, acetylcholine (1×10^{-7} , 2×10^{-6} and $5 \times 10^{-6}M$) was added to the bath and the force of the twitch contraction was compared to untreated controls. There was no differences in the twitches produced.

The results suggest that lead produced a selective lesion in the nerve and the most likely possibility is that lead decreases the amount of acetylcholine released by nerve stimulation. Experiments are in progress to test this hypothesis.

Methyl mercury and CO vs behavior. Dr. Zoltan Annau (Johns Hopkins University) continued his studies on the effects of mercurials and CO on the behavior of mice. Two conditions of exposure were chosen which would reveal the effect of repeated exposures to relatively low doses of mercury on the behavior of adult animals and indicate whether a suitable behavioral test could be devised that would be sensitive to the effects of mercury before the major neurological symptoms appeared. Efforts were made also to observe whether a single prenatal exposure to a non-toxic dose of organic mercury, administered to the mother, would have detectable effects on the offspring in subsequent tests. The results of the first approach have been clear cut in that when eight adult mice were trained on a complex behavioral task, the repeated weekly administration of small doses of methylmercury hydroxide resulted in a decrement in performance after three weeks of treatment. Cessation of treatment after seven weeks resulted in recovery of performance to control levels by the tenth week. Two of the mice developed gross neurological symptoms by the eighth week and died. None of the other mice showed any symptoms during or after treatment with mercury. In short, results with methylmercury suggest that the effects of chronic low level exposures can be detected before gross behavioral manifestations of toxicity will appear. A more important finding from the point of view of human exposure is that low level maternal exposures can leave permanent deficits in the learning capability of the offspring.

Initial work with carbon monoxide (CO) exposure was concerned with the behavioral effects of acute exposures. Using the electrical self-stimulation of the brain as the behavioral model, animals were exposed to different concentrations of CO for three hour periods. The results indicate that no changes in performance occur until the animals are exposed to 500 ppm CO. In animals prepared with chronic indwelling venous or arterial catheters, blood gas and pH determinations were made during exposure to CO or hypoxic hypoxia. The slow rise in blood carboxyhemoglobin content during these exposures suggested that behavioral observations during more prolonged exposure might be more accurate in describing the effects of CO on behavior. These studies suggest that the effects of CO on the central nervous system are different

from the effects of hypoxic hypoxia. This difference seems to be most evident when considering the rate of electrical self-stimulation of the brain. During the first 12 hours of exposure to mild hypoxic hypoxia, the rate of self-stimulation increases markedly, while during exposure to CO hypoxia, the rates decline. The effect of both conditions on food and water intake seem to be similar, although the effects of hypoxic hypoxia are more severe under equivalent conditions as indicated by venous oxygen tension.

Mutagenesis and Reproductive Toxicology

Cyclophosphamide and Bidrin. Dr. James Gibson has studied the effects of cyclophosphamide on the development of behavioral characteristics in mice. Cyclophosphamide administered i.p. to pregnant mice at doses of 5 or 10 mg/kg on the eleventh day of gestation impaired neither the development of swimming nor the development of the righting reflex.

Bidrin was administered i.p. to pregnant mice at doses of 0.2, or 5 mg/kg/day on days 8 through 16 of gestation. A fourth group of mice was used as uninjected controls. Bidrin treatment significantly increased the percentage of animals that were delayed in parturition but did not affect litter yield (number of pups), postnatal mortality, or body weight up to three weeks of age.

The development of swimming was not affected by bidrin nor was activity as measured by the number of squares entered or the number of rearings (standing on hind legs) in an open field at 3 weeks of age.

Mice treated with cyclophosphamide on the day of birth were significantly delayed in their development of swimming ability as compared to water injected controls between the ages of 6 and 21 days. Mice treated with doses of 20 and 30 mg/kg achieved control performance by ages 14 and 15 days, respectively, while the 45 mg/kg group was still impaired at 21 days of age.

In a second test of preweaning behavior, cyclophosphamide reduced the percentage of mice that righted themselves within 15 seconds after they were placed on their back. The χ^2 for the percentage that righted was significant on days 6 to 9 of age but not on days 5 or 10 to 12. While the percentages were lower than controls in the 20 and 30 mg/kg groups, the greatest effect was in the 45 mg/kg group and this probably accounted for the significant χ^2 .

These tests show that cyclophosphamide, but not Bidrin, affects the behavior of mice whether administered to the mother or to the newborn.

DDT and other pesticides. Dr. Ronald Gellert (University of Washington) has examined the effects of various pesticides and chemically-related agents on developing neuroendocrine and reproductive processes.

Previous work demonstrated persistent estrus syndrome (PES) in rats following neonatal treatment with o,p'-DDT. This prompted an investigation of other DDT isomers and homologs. Newborn female rats were injected with 1 mg of each agent on the second, third, and fourth days of life, or pregnant dams were

given daily gavages with 10 mg of specific DDT homologs from the fifteenth to the nineteenth days of pregnancy. Eleven percent of untreated animals developed persistent vaginal estrus (PVE) by seven months of age and by 8 1/2 months 22 percent were anovulatory as seen by the absence of corpora lutea. This is regarded as a normal process of aging of the reproductive system and has been described by other investigators. Therefore, the treated animals must be compared against this background of PVE and anovulation in the control group.

Rats treated as neonates with p,p'-DDT and o,p'-DDE did not develop PVE or anovulation by 8 1/2 months of age in spite of the fact that the latter has been shown by others to be slightly estrogenic. An unexpected finding was that o,p'-DDD, found in other laboratories to be devoid of estrogenicity, induced PVE and anovulation in 58 percent of the treated rats. Agents usually effective in inducing this syndrome are either estrogenic or androgenic and act via hypothalamic or other CNS mechanisms. The action of o,p'-DDD cannot be explained on the basis of sex hormone-like activity.

Homologs with well-demonstrated estrogenic activity such as o,p'-DDT, DDA' and o,p'-DDE, as well as non-estrogenic homologs p,p'-DDT and o,p'-DDD produced no PVE in the offspring of dams treated during pregnancy.

A number of pesticides were tested for estrogenic activity by injecting them in oil into weanling rats and weighing the uterus 24 hours later. Those compounds showing estrogenic activity were Kepone at a dose of 10 mg/kg body weight and carbaryl at a dose of 1 mg/kg. Those agents devoid of activity at doses up to 100 mg/kg were aldrin, dieldrin, mirex and hexachlorophene.

The structural similarity between DDT and hexachlorophene (HCP), the active antibacterial agent in PhisoHex cleanser, prompted an investigation of the effects of this agent given to neonates. Furthermore, the well-known CNS lesions induced in human and animal neonates suggested that this agent may alter normal differentiation involved in regulating reproductive function. Therefore, male and female rats were washed with PhisoHex cleanser (3 percent HCP) for the first eight days of life. Controls were untreated or were washed with Phisoderm, a topical cleanser without HCP. The onset of puberty in female rats as indicated by vaginal opening age was normal in the HCP-treated animals. Estrus cycles and fertility were also unaffected. Fertility was tested in males at seven months of age by exposing them for two weeks to two females of proven fertility. Only 40 percent of the HCP-treated males sired progeny as compared to 100 percent in the Phisoderm and 75 percent in the control groups. In order to determine if inappropriate mating behavior played a role in the reduced fertility rate, the males were exposed for a standard period of time to female rats primed 48 hours previously with 100 ug estradiol benzoate and exhibiting estrus behavior; i.e., darting, hopping, ear wiggling and lordosis. Although the PhisoHex-treated rats demonstrated normal intromission and mounting behavior, significantly fewer treated rats (3 out of 13) ejaculated. Since androgen levels play an important role in male sexual behavior, serum testosterone was determined but no significant differences among the groups were apparent.

Bidrin, Paraquat and Dinoseb. In further studies on the perinatal toxicity of pesticides Dr. James Gibson (Michigan State University) found that the perinatal toxicity of bidrin and the effect of the insecticide on the developmental patterns of brain acetylcholinesterase (AChE) and choline acetyltransferase (ChAc) were determined in mice. Bidrin was concluded to have a low potential for induction of terata in mice even though it transiently reduced embryonic or fetal AChE activity to 1.8 percent of control levels following acute administration on day 11 of gestation. Furthermore, prenatal exposure to bidrin does not alter the development of AChE or ChAc. No changes in the pattern of AChE isozyme development were noted in bidrin poisoned fetuses using polyacrylamide gel electrophoresis.

In some other studies, mice were treated i.p. with Dinoseb at doses of 0-18.8 mg/kg/day on days 10, 11, and/or 12 of gestation. These treatments were preceded by 24 to 48 hours food deprivation. Dinoseb-induced external soft tissue and skeletal anomalies were increased by 24 hour food deprivation while 48 hour deprivation had no effect.

Experiments in which pregnant mice received either phenobarbital or SKF-525A before Dinoseb treatment show that SKF-525A potentiated and phenobarbital inhibited resorptions and reductions in fetal body weight induced by Dinoseb. Food deprivation for 24 hours slowed and phenobarbital hastened disappearance of ^{14}C -Dinoseb from plasma. Deprivation for 48 hours and SKF-525A pretreatment did not affect disappearance of Dinoseb from plasma but respectively increased and decreased the rate of disappearance from liver. Alterations in Dinoseb induced embryo toxicity and teratogenicity by the pretreatments may have been related to an alteration in the rate of disappearance of Dinoseb from the mother.

Administration of paraquat to mice, 1.67 and 3.35 mg/kg i.p. or 20 mg/kg orally, daily on days 8 through 16 of gestation, induced no significant teratogenic effects. Radioactivity reaching the mouse embryo after i.p. or p.o. administration of ^{14}C -paraquat to pregnant mice on day 11 of gestation was low. In fetal organ distribution studies ^{14}C -paraquat was not retained in mouse lung tissue when compared to liver and kidney in contrast to a significant retention of paraquat in one day old mouse lung and maternal mouse lung. The selective retention of paraquat in lung tissue of postnatal but not prenatal animals implicate the necessity of high oxygen tensions for the development of pulmonary toxicity.

Paraquat perinatal toxicity was examined by addition of 50, 100 and 150 ppm paraquat to the drinking water of pregnant mice starting on day eight of gestation with continued exposure 42 days postnatally. Pregnant mice receiving 150 ppm paraquat died before parturition. Paraquat, 50 and 100 ppm, in the drinking water had no effect on growth rate of neonatal animals up to 42 days of age. However, the average percent mortality on a per litter basis was significantly increased by 100 but not 50 ppm paraquat. Accumulative mortality was 70 percent by 42 days after birth. Control lungs 42 days after birth showed no signs of pathological lesions, but lungs of mice treated with 100 ppm paraquat throughout development showed extensive consolidation, collapse of alveoli, edema, and thickening of the intra-alveolar septa. The LT_{50} of

42 day old control mice in a 100 percent oxygen environment was 160 hours. Paraquat treatment at 50 ppm throughout development decreased the LT₅₀ for mice in oxygen to 108 hours while 100 ppm decreased the LT₅₀ to 40 hours.

Paraquat. The mode of action of paraquat was studied by Dr. James Gibson (Michigan State University). In paraquat poisoning, clinical observations indicate liver and kidney damage but patients appear to recover from hepatic and renal effects sometime before death by respiratory failure. Thus, the effects of paraquat on hepatic and renal function were determined. Sulfobromophthalein (BSP) clearance was determined in rats 2 and 32 hours following an oral seven day LD₅₀ dose. Two hours following paraquat administration, plasma disappearance of BSP was the same as controls. However, BSP plasma clearance was significantly slowed in animals administered BSP 32 hours following paraquat.

To determine what step in the BSP biliary excretory process was affected by plasma clearance of glutathione conjugated BSP was determined. There was no significant retention of BSP glutathione in the plasma of paraquat treated rats. Furthermore, the plasma disappearance of indocyanine green, an agent handled by the liver in the same way as BSP but without requiring a conjugation step, was not altered. These results suggest that paraquat initiates lipid peroxidation. The reducing equivalents needed to detoxify lipid hydroperoxides are obtained from reduced glutathione. Reduced glutathione is also needed for the excretion of BSP. Thus, as a result of paraquat treatment BSP would have to compete for reduced glutathione. Twenty-four hours following paraquat there was a significant decrease in liver reduced glutathione.

The nephrotoxic effects of paraquat may delay its own excretion, enhancing its toxicity to the lung and other organs. To test this possibility renal function in mice was determined following paraquat. Paraquat affected its own excretion as shown in experiments where animals were treated with an LD₅₀ dose of paraquat 24 hours before receiving a second paraquat dose. Paraquat poisoned mice had a higher plasma concentrations of the chemical after the second dose than controls.

The observed interaction between oxygen and paraquat led to the postulation that paraquat toxicity may be linked to the presence of oxygen and the initiation of lipid peroxidation. A series of in vitro experiments showed that paraquat was reduced by mouse lung microsomes and that in the presence of NADPH and NADPH-cytochrome c reductase, paraquat increased the in vitro peroxidation of rat liver microsomal lipid. Lipid peroxidation was inhibited by superoxide dismutase and the singlet oxygen trapping agent 1,3-diphenylisobenzofuran. It was suggested that paraquat toxicity may be mediated through a transfer of a single electron from reduced paraquat to oxygen to form superoxide ion. These investigations offer an explanation for paraquat enhanced oxygen toxicity.

Lipid hydroperoxides may be detoxified by enzymatic reduction to lipid alcohol by glutathione peroxidase, a selenium dependent enzyme, which utilizes reduced glutathione as substrate; or by the action of vitamin E in detoxifying the lipid free radicals. In accord with this, acute paraquat toxicity in adult

mice was found to be significantly enhanced by selenium and vitamin E deficient diets and diethylmaleate pretreatment. The interactions of paraquat with oxygen and selenium and vitamin E deficiency not only suggests possible environmental interactions that can occur in the presence of paraquat but also provides valuable insight into the mechanism of action of paraquat toxicity.

Dr. Raymond Harbison has continued studies on pesticide-induced changes during the perinatal period. The organophosphorous insecticide, parathion, produced a significant incidence of in utero deaths. Increased sensitivity of the fetus to paraoxon-induced inhibition of cholinesterase appears to be a result of the normally low cholinesterase activity per gram of fetal tissue. Phenobarbital treatment completely eliminated paraoxon from fetal tissue. This treatment alters transfer of paraoxon from maternal to fetal tissue. The critical factor determining parathion-induced fetotoxicity is the circulating level of paraoxon in maternal blood. Maternal plasma cholinesterase remains depressed after phenobarbital treatment, but fetal cholinesterase is normal.

Bidrin induced as great a depression of cholinesterase activity in both placental and fetal tissue when compared to parathion. However, Bidrin did not induce a significant incidence of fetal resorptions. Reversibility of Bidrin-induced cholinesterase inhibition seems likely to account for lack of increase in fetal resorptions. Thus, depression of fetal cholinesterase levels is not sufficient to induce fetal death, but irreversible inhibition or prolonged depression of fetal cholinesterase represents a hazard to normal fetal development.

Pesticides in general usually considered to be more toxic in the newborn because of a decreased metabolic detoxification system in newborns. However, the chlorinated pesticides, heptachlor, chlordane and DDT, are less toxic in the newborn when compared to the adult, but phenobarbital pretreatment potentiates their toxicity in the newborn. Parathion, on the other hand, is more toxic in the newborn when compared to adult, but phenobarbital pretreatment antagonized the toxicity of this cholinesterase inhibitor.

The existence of an acetylcholine (AChE)-choline acetyltransferase (ChA)-acetylcholinesterase (AChE)-like system in sperm has been proposed as a result of preliminary data obtained in this laboratory. Sperm ChA activity seems necessary for normal sperm function and depression of sperm ChA activity induces sterility. ChA activity has been found in sperm of all species evaluated to date, including man. Since ChA synthesizes ACh, compounds affecting sperm ACh levels can be expected to influence male fertility. Pesticides affecting sperm ACh levels may influence male reproductive efficiency. Also, ChA inhibitors may be a new potentially useful class of male antifertility compounds.

In further studies by Dr. Paula Stern (Northwestern University) on effects of DDT on the developing skelton, it was found that when nursing mice were fed diets containing peanut oil plus 100 or 200 ppm DDT from the date of delivery until they stopped nursing, the calcium content of their milk was not significantly different from that of untreated nursing mice.

Effects of DDT on calcium absorption were also examined. Adult male rats were fasted overnight and given either peanut oil or 50 mg/kg DDT in peanut oil by gastric tube. Twenty-four hours later an intestinal loop was isolated in vivo and injected with 1 ml of saline containing 10 μ Ci ^{45}Ca and 1 mg CaCl_2 . One to two hours later, blood was collected by cardiac puncture. The blood, intestinal lumenal contents and ashed segments were analyzed for ^{45}Ca . In five experiments, each with an N of at least four DDT-treated and four control animals, DDT did not significantly alter the percent Ca absorbed.

In view of the apparent association of calcium transport with Ca-ATPase activity and the reported effects of DDT on Na, K-ATPase , studies were undertaken on Ca ATPase . At 37°C , no effects of DDT were obtained. At 22°C 10^{-5}M DDT produced a 34 percent decrease in enzymatic activity, and 10^{-4}M DDT produced a 46 percent decrease. This was the maximum effect obtained. With the intestinal microvilli 0.6 mM calcium was optimal. A maximal inhibitory effect of 30 percent was obtained with either 10^{-4}M DDT or DDE. The skeletal muscle preparation was found to have a calcium optimum of 0.6-1.0 mM and a Mg optimum of 4 mM. Hydrolysis was linear at times up to 20 minutes. Peak rates of hydrolysis were obtained with ATP concentrations of 1 to 4 mM. DDT, added in absolute ethanol, was inhibitory at concentrations of 10^{-6}M and greater. Maximal inhibition (31 percent) was obtained with 10^{-5}M DDT.

The finding that mercuric chloride had effects similar to those of DDT on eggshells (Stoewsand, G.S. et al.) led to examination of the effects of mercury-containing compounds on the Ca-Mg ATPase . Methyl mercury was inhibitory at concentrations of 10^{-7}M and greater. Concentrations of $2 \times 10^{-6}\text{M}$ and higher inhibited the enzymatic activity 85 to 95 percent. Low concentrations of methyl mercury appeared to increase activity slightly and 10^{-8} and $5 \times 10^{-8}\text{M}$ methyl mercury antagonized the inhibitory effects of DDT. At higher concentrations, the inhibition produced by combined treatment was greater than that with either agent alone, but was less than additive.

Heavy Metals

Methylmercury. Work by Wynetka Reynolds (University of Illinois) using chronic doses of methylmercury in monkeys has previously shown that although methylmercury crosses the placenta slowly from mother into the fetus, return transfer from fetus to mother is even slower than a fetomaternal concentration gradient persisting above 25:1 after many hours. Methylmercury is bound to a greater extent by fetal than maternal erythrocytes which may partially account for fetal retention of organic mercury. Thus, the placenta affords a partial impediment to acute transfer of organic mercury. However, it was confirmed by chronic studies that retarded fetomaternal transport of organic mercury could lead to fetal accumulation. Methylmercury chloride plus a tracer of ^{203}Hg was administered weekly to pregnant monkeys beginning as early as the sixth week of gestation. Newborn levels of ^{203}Hg were twice those in maternal blood. Total mercury in maternal and neonatal blood samples and in total body homogenates was measured. Newborn blood levels of organic mercury averaged 1.28 ng/ml, while maternal levels were 0.56 ng/ml. Mercury content of fetal carcasses averaged 10 ng/gm of primarily organic mercury, over three times the maternal levels of 3 ng/gm. Thus, although the placenta tends to

protect the primate fetus from this poison on an acute basis, chronic exposure to methylmercury results in fetal accumulation of the substance far above maternal levels.

Maternal poisoning was observed at 1 to 5 mg/kg/week after 7 to 13 weeks of exposure as evidenced by symptoms ranging from mild tremor and ataxia to total paralysis, blindness and death. At dosages of 0.5 mg/kg/week for at least 13 weeks, mothers exhibited no overt clinical signs of neurologic damage. One infant of three whose mother received 0.5 mg/kg/week and all infants whose mothers received 1.0 mg/kg/week for 13 weeks, exhibited ataxia and an inability to cling to the mother for suckling. Thus, 0.5 mg/kg/week of methylmercury would appear to be approaching the minimal maternal intake level during primate pregnancy at which overt signs of mercury poisoning will follow in the newborn.

To assess risk in humans, mercury levels in 100 consecutive maternal-cord pairs and 20 breast milk samples were surveyed. Mercury was detectable in 60 percent of cord bloods (maximum level 6 ng/mg) and 40 percent of milk samples (maximum level 4 ng/ml). Proportions of inorganic and organic mercury could not be discerned at such low levels but the similarity of values in maternal and cord bloods and in maternal blood and breast milk suggests that mercury in these patients was principally inorganic.

Dr. Karle Mottet has done extensive work in methylmercury teratogenesis in a number of species. He now has assays from a total of 113 human autopsies to determine body burden in the human population. The subjects range in age from third month of gestation to 90 years. A total of 758 assays were carried out in duplicate on 14 organs and tissues. Over 70 percent of the determinations had a mercury burden of less than 0.25 ng/g of tissue wet weight and only 10 percent were above 0.75 ng/g. The mercury burden of fetal organs and tissues was more uniform than that observed during post-natal life though the level was slightly higher than it was during the first and second decade of life. For the majority of individuals there was no progressive increase in the mercury burden in the tissues with advance age. No cases of unusually high levels of mercury were encountered.

Efforts during this report period were directed also to the completion of studies on rats. Methylmercury, dissolved in a balanced salt solution, was injected subcutaneously into pregnant rats in four divided doses (at plugging and at 4, 8, and 12 days of gestation). The dose of methylmercury given was adjusted to cover a broad range of mercury burden for pregnant females from 60 to 400 times the adult human tissue burden recorded above. The maternal liver mercury burden served as an index of body burden and ranged from four to over 100 ng/g.

Pregnancy was usually interrupted on the twentieth day of gestation and the fetuses were evaluated with respect to the presence or absence of anomalies, body weight, fetal death, numbers of pregnancy sites and mercury assay. No specific anomalies were observed to occur in the fetal rat; however, with higher dosage, a decrease in the weight of the newborn is detected and subcutaneous edema is present. Assay of the total protein substance of the fetuses revealed a 10 to 20 percent decrease in body protein which was dose

related. To rule out the possibility of a metabolic effect on the dam rather than the fetus this experiment was repeated on chick embryos where transplacental nutritional influences are not possible. Decreased protein synthesis and weight of embryo was observed to be comparable to that observed in the rat fetus.

The ontogenesis of behavioral characteristics was studied in six litters of rats which had been exposed to mercury during gestation and nursing sufficient to produce moderate runting but no newborn deaths. The experimental and control rats were kept under identical conditions in the same animal quarters, cages and environmental stimulation. The onset of such behavioral features as nursing, counter-twist, face wash, walking, running, reaction of clicking sounds, fur licking, sniffing, chewing, jumping, interest in food, climbing of walls and noticing observer were observed throughout a 30-day period beginning at birth. The swimming reflex was tested at the end of the observation period. No detectable difference between the experimental and control litters in behavioral characteristics were identified during the period of observation.

Pilot studies were begun about a year ago on M. mullata monkeys to further assess behavioral changes that chronic low level doses of mercury may produce. These investigations were undertaken to lay the groundwork for subsequent studies using pregnant female monkeys to study both the morphologic and behavioral development of methylmercury exposed monkeys. The monkeys were exposed to some exploratory neurophysiological and psychological tests to determine the best avenue of approach to further detailed behavioral testing. Of the experimental monkeys, one was maintained at a mercury blood level of about 0.5 ng/g of blood, two were maintained at approximately 1.0 ng/g and one was maintained at 4.0 ng/g. These produced liver levels of mercury of 7, 14, 35, and 51 ng/g respectively. The pathologic studies on these monkeys are sufficiently complete to indicate that there are some subtle changes heretofore not described in the human and laboratory animal investigations. Most important among these are the presence of myofibrillar degeneration in some cardiac muscle cells similar to that seen in some types of cardiomyopathy produced by catecholamines, potassium, or magnesium deficiency or excess cobalt. A ballooning degenerative change was seen in the Paneth cells of the small intestine. Excessive skin pigmentation was seen in the experimental animals. The distribution of the brain lesions in these monkeys was different than that seen in the human. There were numerous small patches of neuronocytolysis in the insula, temporal pole and visual areas of the cerebral cortex. Numerous other small foci of similar degenerative changes were scattered throughout the cortical substance with least evidence of change around the central sulcus. Cerebellar lesions such as those described in the human were not found. The cage behavior of the monkey with the highest dose was distinctly abnormal, resembling that of human clinical symptoms.

Dr. Virgil Ferm and his colleagues (Dartmouth College) have conducted extensive studies on the teratogenicity of heavy metals such as Mn, Cd, and Pb. A study of the possible teratogenic effects of Mn has been completed. These studies included experiments on the permeability of the early hamster placenta (day eight to nine) to the metal. Previous studies by others have shown that

deficiencies of this metal in the diets of pregnant mice and minks induced a rather specific defect in the otic capsule of the fetus. This defect could be corrected by the simple supplementation of Mn during the critical stages of otolith development. Dr. Ferm has shown that excess Mn injected intravenously into pregnant hamsters does not cause any increased incidence of developmental malformations in the fetuses. However, Mn does cross the placental membrane of the nine day old hamster embryo and detectable amounts can be found in embryonic tissues and in placental tissues. Special attention to the yolk sac concentrations suggest that Mn, like Zn, is not greatly concentrated in this placental membrane. A comparison to concentration ratios for the teratogenic metals, Cd and Hg, however, shows that the yolk sac placenta apparently traps these two teratogens in great quantity.

A study comparing four different routes of administration of mercuric acetate to the hamster, i.e., intraperitoneal (IP), intravenous (IV), oral (O) and subcutaneous (SC) was completed. In all cases embryotoxicity was manifested by an increased incidence of resorbed embryos as well as living embryos which were small edematous and exhibited a delay in growth. The following sequence of decreasing effectiveness in producing embryotoxic effects was also demonstrated, $IP > IV > SC > O$. In addition, an interesting syndrome of cardiac and non-cardiac malformations was detected in embryos from the oral and subcutaneous experimental groups. The major features of this syndrome include ectopia cordis, defects in the ventral body wall, distorted heart ventricles and generalized embryonic edema.

An analysis of data from experiments on the externally visible teratogenic effects of ytterbium chloride indicated that this rare earth element was embryocidal but produced relatively few malformations. Rather than publish this information, the study was extended to include an analysis of possible internal malformations by several different techniques. This additional work has just been completed and indicates that many parts of the developing skeletal system are damaged by ytterbium.

Results of a series of experiments on the embryopathic effects of chromium have recently been completed. These studies demonstrate that at the dosage range used, i.e., 5 to 10 mg/kg, chromium trioxide is embryocidal as indicated by the increased embryonic resorption rate but produces very few specific malformations. This work on Cr was actually the initial phase of a continuing study on the combined teratogenic effects of Cr and Pb. Work to date on this project indicates that the combined effect of these two metals on the hamster embryo is additive. Thus, the major teratogenic effects are edema and growth retardation produced by the Cr as well as the lead-induced abnormalities of the sacral region and tail bud. Further work on the characterization of the underlying skeletal malformations is in progress.

A preliminary comparison of the teratogenic effects of acetazolamide with those of Cd (cadmium sulfate) has shown that it is possible to produce postaxial forelimb deformities in the mouse with Cd that have exactly the same appearance as those produced by acetazolamide treatment. However, Cd can produce defects of either right or left forelimb as well as other malformations. There is some evidence that susceptibility to the teratogenic effects of Cd

is genetically determined. If this turns out to be the case breeding tests will be done to find if mice genetically resistant to the teratogenic effects of Cd are also genetically resistant to acetazolamide.

Intravenous administration of inorganic lead salts to pregnant hamsters early in gestation results in a high frequency of in utero death as well as the production of specific sacral-tail malformations in surviving offspring. A comprehensive histological analysis of embryogenesis in the treated hamsters is nearing completion.

Arsenate. A study of the genetic and other effects of arsenate in plants is being conducted by Dr. Larry Nooden (University of Michigan). He has shown that the roots of fruiting soybeans and tomato plants (grown in mineral solutions) readily take up ^{74}As -arsenate. Also like the corn seedlings, the concentration of ^{74}As inside the roots is much greater than outside, thus uptake is probably active, but this should be checked with metabolic inhibitors. A substantial proportion (10 to 30 percent) of the arsenate taken up is bound to 80 percent ethanol-insoluble material in the roots of tomatoes and soybeans, and most of this bound ^{74}As is not removed by the following sequence of washes at 40 C; 80 percent ethanol (1x), 5 percent TCA (3x), 80 percent ethanol (3x) and CHCl_3 -methanol (2:1; v/v) (1x).

Chromatographic analyses show that after 24 hours of incubation essentially all of the 80 percent ethanol-soluble ^{74}As from tomato roots is arsenite or arsenate, and no other soluble derivatives occur in significant amounts. The relative amounts of arsenite and arsenate vary depending on the part of the plant; i.e., mostly arsenite in the lower parts and mostly arsenate in the upper parts, including the fruit.

After working out the dose-response relationship for the effect of arsenate on seed viability, a pilot study was made to see if the highest dose of arsenate (10^{-2}M) which does not reduce seed viability could alter the mutation rate. Seeds treated with 10^{-2}M arsenate gave rise to chlorophyll mutants at a frequency of about six in 10,000 plants (by contrast none were found in 13,000 H_2O -treated controls). Since these mutants are unable to produce seed, they could not be analyzed genetically. When the treated plants (which looked normal, green) were allowed to self pollinate, mutations segregating out in a 3:1 manner characteristic of recessive nuclear genes were found. These chlorophyll mutations seem to be present in about 1 percent of the individuals growing out of arsenate-treated seeds, but none were observed in 10,000 individuals treated with water only.

A line of barley carrying balanced lethals has been obtained which will facilitate measurement of the effect of arsenate on cross-over rate.

Dr. Robert Chang (University of California, Davis) has continued studies on the leucocyte transforming agent from mononucleosis patients and on other carcinogenic agents. The excretion by mononucleosis patients of a filtrable agent with the capacity of transforming human leucocytes into rapidly and persistently multiplying lymphoid cells has been confirmed by Pereira and her associates (Lancet (i) p. 710, 1972) and by additional experiments by

Dr. Chang in which 23 of 27 mononucleosis patients and 3 of 13 apparently healthy medical students were found to excrete this transforming agent in the upper respiratory secretion. Several persons are still excreting this agent 6 to 12 months after recovery from mononucleosis.

Characterization studies of the agent show that it is inactivated by heat (60° C 30 minutes) and ultraviolet irradiation, but not by ether, DNase or ether followed by DNase. Human sera positive for EBV antibody failed to neutralize the transforming activity. Cells transformed by the agent did not contain EBV antigen. This suggests the transforming agent is a virus not described previously.

Attempts to transform human cells with chemicals and X-irradiation continue to yield negative or inconclusive results. To date the following chemicals were found incapable of transforming amnion cells and neonatal leukocytes: 20-methylcholanthrene, 1,2,5,6-dibenzanthracene, 3,4-benzo-a-pyrene, 9,10-dimethyl-1,2-benzanthracene, 4-nitroquinoline-N-oxide, N-nitrosodimethylamine, ethionine, cadmium, arsenic, urethan, 2-aminopurine, 5-bromodeoxyuridine, ethylmethanesulphonate, nitrosoguanidine and proflavin.

Freeman, et al., described the enhancement by murine leukemia virus of the transformation of rat embryo cells by diethylnitrosamine. Their findings have been confirmed and extended to include mouse and hamster cells treated with a variety of carcinogens. Numerous experiments designed to determine if human cells infected with Raucher leukemia virus are susceptible to transformation by chemical carcinogens have been completed. Again, all results were negative.

To date, Dr. Chang has not been able to show conclusively whether X-ray transforms human amnion cells. The main difficulty lies in the fact that transformed foci also appear in control cultures (at a lower frequency). A very large number of experiments is needed to determine if the difference between X-irradiated and control cells is statistically significant.

Dr. William Lee (Louisiana State University) has conducted an extensive genetic study of environmental mutagens. In *Drosophila*, the mutation rate of progeny from matings 1 to 2 days after removal from EMS were compared with progeny from matings on the fifteenth to twenty-fifth day after removal from EMS should have all developed from cells that were spermatogonia at the time of treatment. It was found that 24 recessive lethals in 2,477 X-chromosomes tested (1.0 percent + 0.4 percent) were in spermatogonial cells at the time of treatment, whereas 69 sex-linked recessive lethals from 271 chromosomes tested (25 percent + 6 percent) were in spermatozoa or late spermatids at the time of treatment. Concurrent controls gave 1/1324 and 1/1006 sex-linked recessive lethals to chromosomes tested in matings during days 2 to 3 and 15 to 25, respectively. This is in agreement with the previously published accumulated controls of this stock of 22/22169 (0.1 percent). The data show a significant increase in the mutation rate following treatment of spermatogonia. Attempts are now being made to develop methods to measure the initial alkylation in spermatogonial cells.

Dr. Andris Kleinhofs (Washington State University) has studied the mechanism of azide mutagenesis. Earlier experiments in this laboratory showed that azide is specific for base-substitution mutations. To differentiate between transitions and transversions and determine if a specific base is attacked, experiments were performed with the Yanofsky tryp- E. coli strains characterized by amino acid sequence analysis of the tryp A protein. The mutants tested include one A $\rightarrow$ T transition, three G $\rightarrow$ A transitions, one G $\rightarrow$ C transversion, two G $\rightarrow$ T transversions, one GA $\rightarrow$ AT double mutant, and three -1 or -2 frameshifts. Only the G $\rightarrow$ T transversions have shown a response to NaN₃.

An experiment with barley was performed to determine if the efficiency of azide as a mutagen in eukaryotes was related to DNA replication. Since the exact time of DNA replication in the embryonic shoot of a barley seed germinating under standard conditions is not known, the azide was applied to seeds following different periods of presoaking between 0 and 24 hours. In practice, the azide was applied at different stages of the cell cycle in the embryonic shoot. It was found that frequencies of chlorophyll-deficient mutations increased up to 12 hours presoak and then decreased. The peak mutation frequency of 80 percent on an M₁ spike basis is much higher than induced by any of the many other mutagens applied to barley. Other experiments have suggested that DNA may be replicating in root or shoot meristems about 12 to 16 hours after initial intake of water. Thus, the results suggest that azide may be a more efficient mutagen on replicating than on non-replicating DNA.

Treatment of bases, nucleosides, or nucleotides with azide under mild conditions (1 M NaN₃, pH 4.0, room temperature) has failed to show any detectable (by thin layer chromatography) modifications to date.

Dr. Marvin Friedman (Medical College of Virginia) has continued work on the nitrosation of naturally occurring amines and amino acids. When 1.0 M dimethylglycine was incubated at 37° C with 1.0 M sodium nitrite for 45 minutes, there was marked nitrosamine production. The ratio of nitrososarcosine to dimethylnitrosamine was pH dependent ranging from 30.3 at pH 1.0 to 8.4 at pH 3.5. Gastric synthesis of nitrososarcosine was simulated by injecting mouse stomachs with dimethylglycine-1-C¹⁴ either alone or in combination with sodium nitrite.

The organ distribution of nitrososarcosine-1-C¹⁴ was determined after oral administration of 1 uCi of the nitrosamine. At three hours after administration the bulk of the nitrosamine was still in the stomach and intestine with significant amounts appearing in the colon. Peak levels occurred in liver, kidney and skeletal muscle by three hours. At four hours, radioactivity was decreasing except in the bladder which went from 1280 cpm to 30,614 cpm. By five hours, the bulk of the radioactivity appeared in urine. The only visceral organ retaining significant amounts of nitrososarcosine was liver. Even at 16 and 24 hours, liver levels were markedly elevated with activity elsewhere only appearing in urine, feces and only slightly in the colon. It was apparent that nitrososarcosine was bound in the liver. Nitrososarcosine also appears to penetrate the brain and testes.

Results of experiments testing mutagenicity of sodium nitrite and dimethylamine indicated that sodium nitrite may suppress dimethylnitrosamine toxicity. These observations were confirmed by elevation of dimethylnitrosamine LD₅₀ by oral administration of sodium nitrite (100 mg/kg). Sodium nitrite (100 mg/kg) inhibited dimethylnitrosamine mutagenicity in the host mediated assay when administered as long as 90 minutes before dimethylnitrosamine. Levels as low as 10 mg/kg inhibited dimethylnitrosamine mutagenicity. In subacute feeding studies lasting eight days, dietary levels of sodium nitrite of 0.25 percent also resulted in significant inhibition of dimethylnitrosamine mutagenicity. This response was shown to be metabolic in nature since high acute levels of sodium nitrite (100 mg/kg) had no effect on the mutagenicity of BCMU, a nitrosourea which does not require metabolic activation. Paradoxically, in preliminary studies, oral administration of sodium nitrite had no effect on methylation of mouse liver RNA or protein by C¹⁴ dimethylnitrosamine.

Dr. Walter Troll and his colleagues (New York University Medical Center) have continued studies aimed at correlation of carcinogenicity and mutagenicity. Various genetically well characterized microbial strains have been used in the experiments in an effort to identify the type of mutation(s) most closely related to carcinogenesis. The tester set of Escherichia coli and Salmonella typhimurium consists of nutritionally deficient mutants which have been assigned specific base-pair changes at the mutant site. These include three tryptophan requiring mutants of E. coli which have been assigned the substitution of adenine-thymine by the amino acid replacement data of Yanofsky; a tryptophan requiring strain of Yanofsky with an adenine-thymine transversion; numerous guanine-cytosine transition mutants of E. coli strain 15; and sets of deletion mutants of S. typhimurium from Dr. Bruce Ames. Tests with these strains have shown that the carcinogenic alpha haloethers, chloromethyl-methyl ether and bis-(chloromethyl) ether are capable of producing base-pair deletion as well as base-pair substitution types of mutations. Further, other direct-acting, carcinogenic halogenated compounds related to the halo ethers, dimethylcarbaryl chloride, epichlorohydrin, perchlorocyclobut-2-enone, monochloroacetaldehyde diethyl acetal, 1,4-dichlorobutene-2, hexachloroacetone, chloropentafluoroacetone, dichlorotetrafluoroacetone, and trichlorotrifluoroacetone, have been found to produce both types of mutations.

Bladder cancer in man and dogs exposed to the carcinogenic aromatic amine, 2-aminonaphthalene most likely occurs through the excretion of urinary metabolites. This laboratory has identified one of these metabolites in the urine of man and dog to be bis-(2-amino-1-naphthyl) phosphate. Mutagenicity experiments with the bis-phosphate metabolite in both yeast and bacteria indicate that this compound is incapable of inducing petite mutations (mitochondrial) in yeast or back-mutations in bacteria. However, these microorganisms exposed to the suspect compound in Udenfriend's hydroxylating medium, resulted in mutation. Thin layer chromatography of the reaction mixture on Avicel plates, using n-butanol/n-propanol/0.1N NH₃ (3:1:1) as solvent revealed the presence of a single new spot ($R_f=0.50$ as compared to the bis-phosphate $R_f=0.80$) having a strong blue fluorescence when viewed under short wavelength UV light. The compound has been identified as bis-(2-amino-1-naphthyl) phosphate.

These results have made it highly desirable to obtain sufficient amounts of bis-(2-amino-1-naphthyl) phosphate for further studies. Recently, Dr. T. Finkelstein, an NCI Fellow working with Dr. Belman has succeeded in synthesizing this compound. Present research with 2-aminonaphthalene and the bis-phosphate indicates that these compounds must be activated to be mutagenic in yeast and bacteria.

DNA Repair. Dr. John Little and his colleagues (Harvard University) has continued studies on factors influencing the repair of potentially lethal radiation damage in growth-inhibited cultures of Chang human liver cells. Normally, the half-time for potentially lethal damage repair was one to two hours. Two types of DNA damage and repair have been demonstrated: (1) an incisional process which probably represents the enzymatic recognition by the cell of radiation-induced base damage (which in order to be excised and repaired first requires the insertion of a break in the DNA chain). This process occurs within one to four minutes of irradiation; and (2) the direct induction of DNA strand breaks due to rupture at the phosphodiester bonds. These breaks are rejoined within five to thirty minutes after irradiation. Evidence was found for these two repair processes in all normal cells studied including a number of human diploid cell strains, several aneuploid lines of human and rodent cells, and two radioresistant hybrid cell lines.

Currently cells variant in these types of DNA repair are being isolated. For example, primary skin fibroblasts have been cultured from a patient who exhibited an unusually severe skin reaction during radiation therapy. These cells also showed increased radiosensitivity in vitro as compared to skin fibroblasts derived from normal individuals. A defective step in the DNA repair process described above has been demonstrated in these cells by the use of alkaline sucrose gradients. This is the first instance whereby it is possible to correlate in vivo and in vitro X-ray sensitivity with a specific molecular process. Clinical radiosensitivity may therefore be a useful indicator in the search for diploid cell strains with abnormal DNA repair mechanisms.

Dr. Raymond Harbison (Vanderbilt University) has continued studies on the embryocidal and fetocidal effects of natural toxins. It was demonstrated that sweet potatoes offered for sale on the market and in food stores contain ipomeamarone and 4-ipomeanol, and that cooking processes do not eliminate the toxins from contaminated sweet potatoes. These findings led to investigation of the toxic and teratogenic roles that these naturally occurring toxicants might play in newborn and pregnant animals.

Similar studies were carried out using rubratoxin B, a hepatotoxic substance formed by the fungus Penicillium rubrum. The mycotoxin, rubratoxin B, is a more potent toxicant than ipomeanine and 4-ipomeanol when comparing their effects on perinatal growth and development. Rubratoxin B induced various nonspecific external and internal malformations. Ipomeanine and 4-ipomeanol-induced anomalies were not incompatible with in utero life and consequently did not produce an increase in in utero deaths. Thus, morbidity is high when considering ipomeanine and 4-ipomeanol, but mortality is high when considering rubratoxin effects.

There is a difference in susceptibility to ipomeanine and 4-ipomeanol-induced toxicity when compared at different gestational periods. Lethality induced by rubratoxin B, ipomeanine and 4-ipomeanol was significantly higher in newborn rats when compared with adult male rats.

Rubratoxin B probably decreases activity of liver mixed function oxidase as indicated by the increased hexobarbital sleep time. This suggests a possibly interaction between rubratoxin and drugs or environmental contaminants which could increase the susceptibility to toxic actions of the latter substances.

Dr. Gerald Wogan and his colleagues (Massachusetts Institute of Technology) has continued studies on the toxicity, mutagenicity and carcinogenicity of a number of compounds prominent in foods. They have developed an assay which defines the dose dependent mutagenicity of a test compound for diploid human cells in culture and are optimistic that this assay can be improved with regard to facility of application and that reliable data can be obtained in 10 to 14 days.

They have used an established but contact inhibited C3H mouse cell line to make in vitro carcinogenicity testing a part of this overall program and have used this tool in research to study the possible cocarcinogenic effects of various food components and additives. At the present time, most of this work is in the exploratory phase in which development and refinement of assay systems occupies a major portion of the total effort.

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